

# MICRORNA EXPRESSION WITHIN PERIOVULATORY MURAL GRANULOSA CELLS

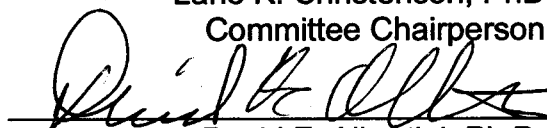
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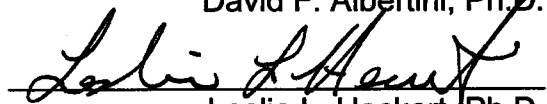
Stephanie D. Fiedler

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Physiology and the Graduate Faculty of the University of Kansas in partial  
fulfillment of the requirements for Master of Science

Thesis Committee:

  
Lane K. Christenson, PhD  
Committee Chairperson

  
David F. Albertini, Ph.D.

  
Leslie L. Heckert, Ph.D.

  
Michael W. Wolfe, Ph.D.

Date Defended: November 14, 2008

The Thesis Committee for Stephanie D. Fiedler certifies  
that this is the approved version of the following thesis:

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Thesis Committee:

  
Lane K. Christenson, PhD  
Committee Chairperson

\_\_\_\_\_  
David F. Albertini, PhD

\_\_\_\_\_  
Leslie L. Heckert, PhD

\_\_\_\_\_  
Michael W. Wolfe, PhD

Date Approved: \_\_\_\_\_

*It is with the greatest love and affection that  
I dedicate this work to my family...*

*To my son Zachary  
Thank you for your patience and understanding. You are mature  
beyond your years, and I am so proud to be your mother.*

*and*

*To my husband Todd  
Words alone cannot express my appreciation for everything you bring  
to my life. You are my strength, and I am truly grateful  
for your love and support.*

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I would like to express my appreciation to my mom for always supporting my decisions and to my dad and Blythe for their confidence in me. I am also grateful for the support of my husband's mom, sister and grandparents; Marcia, Sarah, Herb and Maxine, thank you for asking questions and always showing interest in my progress. I would not have been able to get this far without the support of each and every one of you.

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## **Abstract**

Normal ovarian function is an important aspect of reproductive health, and loss of this function can have drastic consequences including infertility as well as an increased risk for diseases like ovarian cancer. The ability to initiate and maintain a pregnancy is dependent on the regulation of oocyte developmental competence, ovulation and corpus luteum function as each makes an important contribution to proper ovarian function. During the periovulatory period, ovulation is initiated as a surge of LH induces granulosa cells to rapidly transform from estrogen producing follicular cells to progesterone producing luteal cells. The molecular mechanisms involved with the follicular to luteal transition have been extensively studied, and many transcriptional changes have been associated with steroidogenic and/or cell differentiation pathways. Comparatively, little attention has been given to post-transcriptional regulatory events during this period despite such events having an apparent role in maintaining the integrity of gene expression.

MicroRNA (miRNA) are highly conserved, 21nt long RNA molecules that offer a way to regulate gene expression at the post-transcriptional level by binding to the 3'UTR region of a target mRNA and either preventing its translation or causing its degradation. MicroRNA have been shown to be involved in cell differentiation in other systems implying that they may also have a role in the changes that occur within granulosa cells following the LH surge. Within this thesis, I discuss several studies designed to look at miRNA

expression in periovulatory granulosa cells. In addition, I describe several methods that can be used to measure levels of miRNA in their mature form as well as techniques used to begin to determine their functional relevance to the processes of ovulation and luteinization.

I hypothesize that specific miRNA are induced by the LH surge, and that these miRNA are involved with regulating translation of specific target mRNA(s) within periovulatory granulosa cells. Using a mouse model, I have shown 13 miRNA to be differentially expressed at 4 hours post hCG, a critical time during the periovulatory period when many changes in gene expression are known to occur. MicroRNA-132 and miRNA-212 were found to be two of the most highly upregulated miRNA at this time point; therefore, I chose to narrow our examination to the characterization of these two miRNA and their potential mRNA targets within the periovulatory follicle. In addition to miRNA studies, I have also begun to look at the gene within which miRNA-132 and 212 reside. Experiments designed to look at the expression of this gene suggest that it is regulated by LH as well. The results I present here provide the groundwork necessary for studying the function and regulation of miRNA-132 and 212 within periovulatory follicles.

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## **Chapter 1**

### **Introduction and Significance**

#### *Ovulation and Luteinization*

The two main functions of the mammalian ovary are to produce competent oocytes and steroid hormones. The processes of ovulation and luteinization are regulated by a multi-tiered negative feedback system that involves gonadotropins produced by the hypothalamus and pituitary as well as hormones synthesized within the granulosa and theca cells of ovarian follicles. A surge of luteinizing hormone (LH) is the initiating factor for the processes of ovulation and luteinization [1-3].

In response to the LH surge, luteinization of mural granulosa cells begins prior to ovulation and results in their transformation from a follicular estrogen producing cell to a luteal progesterone producing cell. To accomplish these phenotypic and functional changes, several things occur including exit from the cell cycle, external signal response modifications, as well as alterations in gene expression and signal transduction pathways. Cells typically cycle between proliferative and quiescent states in a process that is mediated by cyclins D and E which are regulated by cyclin-dependent kinases (Cdk) [4]. This process is thought to be regulated by Cdk inhibitors p21<sup>Cip1</sup> and p27<sup>Kip1</sup> [5]. The LH surge has been shown to rapidly inhibit cyclin D2 while simultaneously inducing expression of both p21<sup>Cip1</sup> and p27<sup>Kip1</sup> [6, 7].

Together, these events are thought to be associated with the loss of cell cycle regulation that culminates in the absence of GC proliferation during luteinization. Interestingly, knockout studies have shown that GC proliferation is not changed in  $p21^{Cip1 -/-}$  animals, while GC undergo a hyperproliferative state following the LH-surge in  $p27^{Kip1 -/-}$  animals [8]. These results indicate that  $p27^{Kip1}$  may have a vital role in LH-induced GC cell cycle arrest during luteinization.

One of the main signaling pathways in LH-induced granulosa cell differentiation involves the second messenger cAMP [9]. Binding of LH to LH receptors on the cell surface of granulosa cells initiates a signal transduction cascade that leads to the activation of cyclic-AMP-dependent protein kinase (PKA) and the phosphorylation of CRE-binding (CREB) protein. This results in the altered expression patterns of many genes associated with ovulation, cumulus expansion and establishment of the corpus luteum [10]. For example, changes in the expression of progesterone receptor (PR) and peroxisome proliferator-activated receptor gamma ( $PPAR\gamma$ ) are associated with ovulatory events [11, 12], while cumulus expansion is mediated by the induction of epidermal growth factor (EGF)-like factors epiregulin and amphiregulin [13]. These factors have been shown to induce the expression of prostaglandin synthase 2 (*Ptg2*) and human hyaluronan synthase 2 (*Has2*), genes involved with extracellular matrix remodeling [13]. Failure to ovulate in connexin-37 [14], *Ptg2* [15], CCAAT/enhancer binding protein- $\beta$  (*Cebp- $\beta$* ) [16]

and progesterone receptor (*Pgr*) [11] knock out animals indicates the importance of many factors in the ovulatory process and suggests that it is a highly regulated event.

Formation of a functional corpus luteum (CL), an endocrine gland derived from the granulosa and theca cells of an ovulated follicle, is necessary for the establishment of pregnancy and regulation of the estrous cycle [17, 18]. During the period following the LH surge, granulosa cells undergo cell cycle arrest, increase in volume, and begin to fill with lipid droplets [19]. Studies have shown that luteinizing granulosa cells also undergo many changes in gene expression including increases in the expression of scavenger receptor type I, class B (SR-B1) and steroidogenic acute regulatory protein (StAR) [17], factors involved with cholesterol transport and metabolism. Additionally, the switch from estrogen to progesterone production in LH stimulated granulosa cells is supported by increases in the expression of steroidogenic enzymes like cholesterol side chain cleavage cytochrome P450 (CYP11A) [20, 21] and 3- $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) [22] as well as by a decrease in the expression of aromatase [23]. While numerous studies have provided us with important information related to transcriptional regulatory mechanisms that occur within granulosa cells following the LH surge, other mechanisms of gene regulation occur following transcription. These post-transcriptional regulatory

mechanisms are critical for gene expression and are vital to proper tissue and organ function.

### *Post-Transcriptional Regulation and MicroRNA*

Upon initiation of transcription, a number of post-transcriptional control mechanisms are in place to provide a regulatory network which allows mRNAs to be packaged, transported, sequestered and/or destroyed prior to being translated into a protein thus controlling the amount of gene product made into a protein. Regulatory events such as alternative splicing, RNA editing and nuclear transport blockage are often mediated by RNA-binding proteins (RBPs) and occur within the nucleus [10], while other RBP and non-coding RNA mediated mechanisms take place within the cytoplasm [24].

RBPs are *trans-acting* factors that bind to *cis* elements located within the 3' untranslated region (3' UTR) of specific messages and mediate transcript localization within the cytoplasm, as well as mRNA stabilization, translation and degradation [24, 25]. Regulatory specificity is maintained by sequence elements within the 3' UTR. One of the best studied sequence element is the A+U-rich element (ARE), known to be a strong destabilizing element as transcripts containing this structure within their 3' UTR display increased rates of decay [26]. For example, the protein AUF1 mediates mRNA destabilization when bound to the ARE. In contrast, ARE-binding of He1-N1, HuC, HuD and HuR proteins has been associated with mRNA

stabilization [26] indicating a complex regulatory network for ARE containing transcripts.

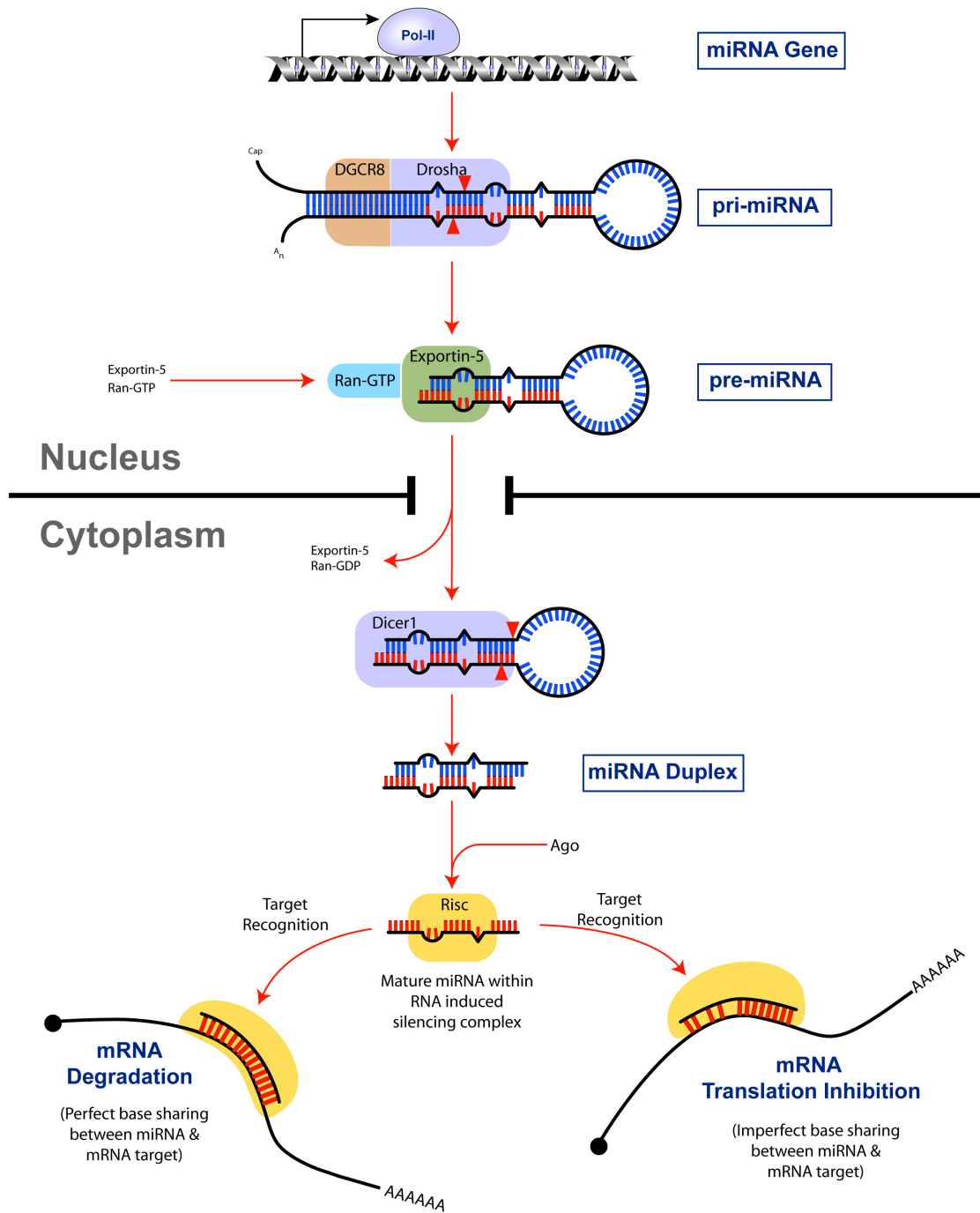
MicroRNAs (miRNAs), another type of *trans-acting factor*, are endogenous ~21nt long non-coding RNA molecules that bind specifically to the 3' UTR region of their target mRNAs and prevent translation [27]. They are predicted to constitute ~1% of the entire human genome and are perhaps responsible for regulating up to one third of all mRNA [28, 29]. Sequences for these small regulatory RNA molecules have been located throughout the genome and include intergenic, exonic and intronic regions of the DNA [30] with more than 3,000 miRNAs having currently been identified in species ranging from plants to humans [28].

Processing of miRNAs occurs in a series of steps (**Figure 1-1**) beginning with the synthesis of a 5' capped and polyadenylated [31] primary miRNA (pri-miRNA) [32, 33]. Pri-miRNAs are cleaved by the RNase III endonuclease Drosha which works in combination with the co-factor DGCR8 to produce a ~60-70 nucleotide (nt) stem-loop intermediate precursor miRNA (pre-miRNA) that contains a ~2nt 3' overhang at the Drosha cleavage site [34, 35]. Drosha's endonuclease activity is specific to double stranded RNA and has been found to be ~11 base pairs from the junction between the stem-ssRNA of the pri-RNA structure. [36, 37]. Following Drosha cleavage, the pre-miRNA stem-loop structure is exported from the nucleus to the cytoplasm by Exportin-5 through a Ran-GTP mechanism [38-40] where it is then cleaved

by Dicer, another RNase III endonuclease. Dicer binds to a region of the pre-miRNA at the base of the stem-loop and cuts both strands of RNA to produce a 3' ~2nt overhang on the opposite strand from the overhang produced by Drosha [33]. This imperfect double stranded RNA duplex is referred to as miRNA:miRNA\* with the miRNA portion referred to as the guide (mature) strand and the miRNA\* portion as the passenger (non-functional) strand [41]. The miRNA:miRNA\* duplex, which is believed to be a transient structure [42], is then preferentially loaded onto the RNA-induced silencing complex (RISC), a structure composed of Dicer, Argonaute proteins, and other RISC associated factors [43]. The strand loaded onto RISC is thought to be the one with the less stable 5' end, while the other strand (typically the miRNA\* strand) undergoes degradation [44, 45]. Interestingly, recent data has shown the existence of functional miRNA on both the 5' and 3' arms for the hairpin pre-cursor; therefore, new conventions for naming these types of functional miRNA include the addition of -5p or -3p to the end of the miRNA notation [46].



**Figure 1-1**



**FIGURE 1-1:** Graphical representation depicting the process of microRNA synthesis and target recognition.

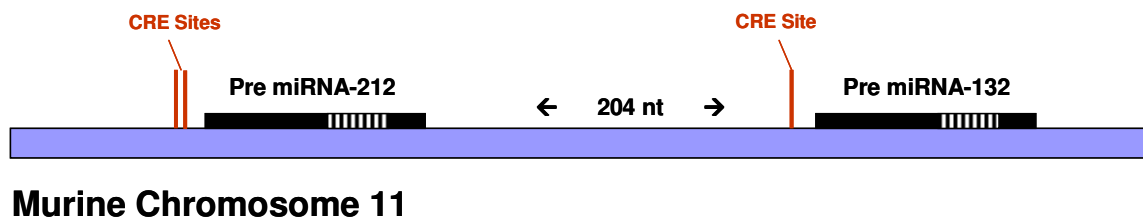
There appear to be two main mechanisms by which miRNAs impose translational regulation on their specific mRNA target(s): repression and cleavage/degradation [28]. The 2-8 position at the 5' end of the mature miRNA, referred to as the seed sequence, is considered to be very important to target recognition [47, 48]. It is the most conserved region of mammalian miRNA [42, 47] and has been shown to be a reliable way to predict targets based on perfect complementary pairing [47]. The determining factor as to whether a mRNA will follow a degradation pathway versus a repression pathway appears to be related to the degree of complementary base pairing that occurs within the 3'UTR. The more perfect the pairing, the more likely the mRNA will be cleaved and degraded [49-51]. This is often the case in plants [52, 53], while mammalian miRNAs typically display less than perfect base pairing with the 3'UTR of their targets [50, 54].

Currently, very little is known about the specific targets for most of the identified miRNAs, therefore the functional aspects of specific miRNAs continue to remain largely elusive [27]. Experimental validation has linked only a handful of miRNAs to a target and/or function [28]. For example, many of the biological roles of miRNAs in nematodes are related to temporal regulation of development [54-58]. Similarly, miRNA function in plants also appears to relate largely to developmental processes [53, 59, 60]. In other species, the role miRNAs play appears to be much more diverse. In *Drosophila*, miRNAs have been shown to control aspects of both cell

proliferation and differentiation. For example, the *bantam* miRNA has been found to target the message for the pro-apoptotic gene *hid*, and thus affect an apoptotic pathway critical to fly development [48]. Furthermore, mammalian miRNAs have been shown to regulate cell growth by acting through a cAMP regulated pathway [61] and have been associated with many other processes including cell cycle regulation, differentiation, apoptosis, oncogenesis and tumor suppression [27].

#### *MicroRNA 132 and 212 and Their mRNA Targets*

The pre-forms of microRNA-132 and 212 are situated 203 nucleotides apart from each other on chromosome 11 of the mouse genome (**Figure 1-2**) within the first intron of a three exon gene (AK006051) that encodes a transcript of unknown function. MicroRNA-132 and 212 share the identical seed sequence (AACAGUC) and contain cAMP regulatory element (CRE) sequences immediately 5' of the sequence that codes for their mature forms. This would suggest that CREB, a transcription factor known to bind to CREs, may bind to and regulate miRNA-132 and 212 expression [61]. This is



**FIGURE 1-2:** Depiction of miRNA-132 and 212 in relation to each other on mouse chromosome 11.

interesting as the cAMP pathway is thought to be one of the main signal transduction cascades induced by LH during the luteinization of granulosa cells [9, 62].

One of the experimentally validated targets of miRNA-132 is p250GAP. Also known as p200RhoGAP/Grit and RICS [63], p250GAP is a RhoGTPase activating protein that has been linked to dendrite differentiation [61, 64]. Rho GTPases are made up of six different classes (Rho, Rac, Cdc42, Rnd, RhoD, and TTF) and are involved in the initiation of signal transduction pathways that result in cellular processes like vesicle trafficking, cytoskeletal reorganization, as well as cell cycle and transcriptional regulation [65], all of which are integrally related to the process of differentiation. RhoGTPases act like molecular switches flipping from an active (GTP bound) state to an inactive (GDP bound) state [66, 67]. Their hydrolytic activity is stimulated by GTPase-activating proteins (GAPs) which mediate cleavage of a phosphate group from the GTP molecule resulting in signal transduction cascade changes [68]. Recently, Vo et al. [61] showed that over-expression of miRNA-132 in a neuronal cell line resulted in a reduction of p250GAP, thus validating it as a target. Inhibition of RhoGAP was also shown to cause an increase in dendrite outgrowth, which was identical to the response seen when miRNA-132 was over-expressed in the same cell line [61].

Much of the work done with p250GAP has been in neuronal tissue, where RhoGTPase activation by RhoGAPs has been associated with actin

stress fiber formation [69] and changes in oligodendrocyte cell morphology [65]. More specifically, RhoGTPase has been shown to reduce the number of dendrite spines [70] via a microtubule based mechanism [71] which promotes the retraction of the cell membrane [66]. In addition to neuronal tissue, p250GAP has also been shown to be expressed in the human ovary [72], and the worm homolog (RRC-1) is known to co-localize to the uterine-seam cells in *C. elegans* [73]. Given the role RhoGTPases have in the control of cell morphology within neuronal cells, it is appealing to consider the possibility of a connection between miRNA-mediated regulation of p250GAP and the morphological changes that occur during luteinization.

Two additional experimentally validated targets of miRNA-132 are methyl CpG binding protein 2 (*MeCP2*) and C-terminal binding protein 1 (*CtBP1*). Klein et al (2007) have shown that these two proteins are regulated by miRNA-132 and are involved with a feedback loop responsible for maintaining homeostatic levels of *MeCP2*, a protein known to play an integral role in dendrite differentiation within the nervous system [74]. *MeCP2* is part of a family of proteins involved with epigenetic regulation. This protein is known to inhibit transcription by both binding to 5`CpG3` pairs that are symmetrically methylated and recruiting corepressor complexes to the DNA resulting in signal transduction changes that alter gene expression [74, 75]. Rett syndrome, an X-linked disease characterized by nervous system developmental defects associated with mental retardation, is known to be

caused by mutations in the *MECP2* gene. Interestingly, neurological deficiencies associated with this disease are diminished in a mouse model following the re-establishment of *MeCP2* protein levels thus validating the link between *MeCP2* and this disease [75].

Similar to *MeCP2*, *CtBP1* is known as a regulator of transcription and also interacts with many transcription factors [76]. Although initially identified as a transcriptional activator of the adenovirus E1A oncoproteins, *CtBP1* is primarily associated with transcriptional repression [76]. As expected, studies have shown the function of many transcription factors to be disrupted in *CtBP1* deficient mice [77]. A recent study has discussed a possible role for *CtBP1* in the regulation of CYP17 expression within adrenocortical cells [78]. Although CYP17 is not expressed within pre-ovulatory GCs, *CtBP1* has been shown to interact with and alter the ability of steroidogenic-factor 1 (SF-1) to stimulate promoter activity. This is interesting as SF-1 is thought to be involved with regulating the expression of many genes within granulosa cells [79]. In addition to its transcriptional regulatory activities, *CtBP1* has a potential link to cytoskeletal activities (including microtubule dynamics) as it is known to bind to PDZ domain proteins responsible for anchoring transmembrane proteins to cytoskeletal components within the cytosol [76]. Given the diverse functional roles of *MeCP2* and *CtBP1*, as they relate to gene expression and cytoskeletal dynamics, combined with the dynamic nature of granulosa cell transcription and phenotypic activity during the

periovulatory period, it is not improbable to speculate that these two proteins could play an integral role in the processes of ovulation and luteinization.

### *Study Significance*

Women's reproductive health care is an area of increasing research interest and proper ovarian function plays a significant role in both reproductive ability and the overall health of an individual. The importance of normal ovarian function is highlighted by the number of women who encounter difficulties achieving pregnancy. Infertility, defined as the inability to get pregnant following a one year trial period, affects millions of women each year as suggested by the incidence of infertility-related appointments scheduled by 2% of women of reproductive age. In addition to fertility issues, the reproductive health of women can be compromised by diseases like Polycystic Ovarian Syndrome (PCOS) and ovarian cancer. PCOS, a treatable disease associated with high androgen levels, abnormal follicle growth, hirsutism and affects 5-10% of women of child-bearing age in the United States [80]. This disease is non-fatal but is associated with a high incidence of infertility as well as other non-reproductive symptoms including insulin resistance, obesity and high-blood pressure. In contrast, ovarian cancer is the most fatal of all female reproductive tract cancers undoubtedly due to a lack of early detection. Typically originating from epithelial cells lining the outer surface of the ovary, more than 21,000 women are diagnosed with ovarian

cancer each year; furthermore, this disease is responsible for more than 15,000 deaths annually [81]. These major reproductive health issues suggest the need for more knowledge about the mechanisms involved with maintaining proper ovarian function.

The recent discovery of miRNA offers the prospect of new and exciting post-transcriptional regulatory mechanisms that may play an integral role in the maintenance of normal cellular processes during the follicular and luteal phases of the ovarian cycle. A growing body of evidence has linked miRNA to cell transformation processes as well as tumorigenesis, but the mechanisms by which miRNAs cause these outcomes are poorly understood. The experiments within this thesis represent a starting place and will provide a sound basis for future studies into the role miRNA play in ovarian granulosa cell function. They will also establish the basic methodological tools that will be essential for dissecting the role of miRNA in ovarian function.



## **Chapter 2**

# **Hormonal Regulation of MicroRNA Expression in Periovulatory Mural Granulosa Cells**

### **ABSTRACT**

MicroRNAs (miRNA) mediate post-transcriptional gene regulation by binding to the 3'untranslated region of messenger RNAs to either inhibit or enhance translation. The extent and hormonal regulation of miRNA expression by ovarian granulosa cells and their role in ovulation and luteinization is unknown. In the present study, miRNA array analysis was used to identify 212 mature miRNAs as expressed and 13 as differentially expressed in periovulatory granulosa cells collected before and after an ovulatory surge of hCG. Two miRNAs, *Mirn132* and *Mirn212* (also known as miR-132 and miR-212), were found to be highly upregulated following LH/hCG-induction and were further analyzed. *In vivo* and *in vitro* temporal expression analysis by quantitative RT-PCR confirmed that LH/hCG and cAMP, respectively, increased transcription of the precursor transcript as well as the mature miRNAs. Locked nucleic acid oligonucleotides complementary to *Mirn132* and *Mirn212*, were shown to block cAMP-mediated mature miRNA expression and function. Computational analyses indicated that 77 putative mRNA targets of *Mirn132* and *Mirn212* were expressed in ovarian granulosa cells. Furthermore, upon knockdown of *Mirn132* and *Mirn212*, a known target of *Mirn132*, C-terminal binding protein 1, showed decreased protein levels but

no change in mRNA levels. The following studies are the first to describe the extent of miRNA expression within ovarian granulosa cells and the first to demonstrate that LH/hCG regulates the expression of select miRNAs, which affect post-transcriptional gene regulation within these cells.

## INTRODUCTION

Ovulation of a mature and viable oocyte and the formation of a functional corpus luteum (CL) are essential for establishment of pregnancy. These events are preceded by a highly orchestrated series of growth and developmental events in the periovulatory follicle that ultimately signals the preovulatory surge of luteinizing hormone (LH) resulting in the initiation of ovulation and CL formation [1-3]. Numerous studies have shown that the LH surge initiates the transcriptional up and down regulation of genes, including cytokines, transcription factors, and matrix-remodeling proteins within periovulatory granulosa cells [6, 82, 83]. Furthermore, the signaling mechanisms whereby the LH receptor transduces this endocrine signal within ovarian granulosa cells is well described [9, 84, 85]. However, post-transcriptional gene regulation with respect to ovarian function [86], outside of oocyte development [87], has been largely overlooked. The recent identification of microRNAs (miRNAs) within somatic tissues, in particular rapidly differentiating cells such as embryonic tissues and cancer cells, has implicated post-transcriptional gene regulation as a central player in development [27, 88-90]. In the adult animal, the cyclic phenotypic changes that take place in the ovary and uterus are some of the more dramatic cellular and tissue changes to occur outside of embryonic development. The structural and functional transformation that takes place as the highly proliferative periovulatory follicular granulosa cells terminally differentiate into luteal cells

occurs exceptionally fast and requires a complete change in the cellular phenotype. The importance of transcription in this conversion is highlighted by the marked number of genes that are transcriptionally regulated during this period [91-93] and by the essential role specific LH-induced genes (e.g. progesterone receptor, CCAAT/enhancer binding protein  $\beta$ , prostaglandin synthetase-2, cyclin D2 [7, 11, 15, 16]) have on the processes of ovulation and luteinization. No singular method has allowed for the global evaluation of post-transcriptional gene regulation as it pertains to ovarian granulosa cell function; nonetheless, it is relatively easy to envision that during the phenotypic transformation of the granulosa cells, follicular transcript turnover and/or the blockade of follicular transcript translation would facilitate the processes of ovulation and luteinization. MicroRNAs provide such a post-transcriptional mechanism whereby large classes of transcripts may be simultaneously and effectively regulated by individual factors [94].

MicroRNAs are highly conserved, ~21nt long RNA molecules that post-transcriptionally regulate the expression of specific mRNAs or groups of mRNAs based on interactions with the 3'untranslated regions (UTR) of specific mRNAs [27]. MicroRNAs are initially transcribed as pri-miRNAs, can be several kilobases in length and contain a characteristic hairpin loop structure [33, 95]. These pri-miRNA transcripts are cleaved by the RNase III endonuclease Drosha which works in combination with its co-factor DGCR8 to produce a ~70 nucleotide stem-loop intermediate precursor miRNA (pre-

miRNA) [40]. Following Drosha cleavage, the pre-miRNA stem-loop structure is exported from the nucleus to the cytoplasm by Exportin-5 through a Ran-GTP mechanism [38] where it is then cleaved by Dicer to produce the mature form of the miRNA [33]. The mature miRNA harbors a sequence of 7 nucleotides known as the seed sequence (bases 2-8 of the miRNA), a region that binds to the 3'UTR of target mRNAs. This mature miRNA, in association with the RNA induced silencing complex, effectively blocks the process of translation and/or causes the degradation of the targeted mRNAs [47, 96]. Moreover, recent observations of Vasudevan et al. [97] illustrate that miRNAs can differentially regulate translation, inhibiting under conditions of active cell proliferation and promoting under conditions of cell cycle arrest. Despite having been shown to be involved with proliferation and cell differentiation in a variety of systems [27, 98, 99], the role of miRNAs within periovulatory granulosa cells is presently unknown. In the following set of experiments, we have identified several hormonally regulated miRNAs within periovulatory granulosa cells. Additionally, we have examined the expression of the two most significantly upregulated miRNAs and have begun to identify downstream targets that may establish their potential role during the processes of ovulation and luteinization.

## METHODS AND MATERIALS

### *Animals and Granulosa Cell Isolation*

Nineteen-day old CF-1 female mice were injected i.p. with 5IU eCG to induce follicular stimulation. After 46 hours, the ovaries were either collected (0 hour time point) or the mice were injected i.p. with 5IU of hCG and ovaries collected at 1, 2, 4, 6, 8 and 12 hours post-hCG. Follicles were punctured with a 30-gauge needle and granulosa cells were collected into ice-cold PBS [100]. Mural granulosa cells were separated from cumulus oocyte complexes using a 50  $\mu$ m Nitex size exclusion filter (Wildlife Supply Company, Buffalo, NY). The mural granulosa cells were then pelleted by centrifugation (770 x g) and resuspended in 500  $\mu$ L of TRI Reagent for subsequent RNA extraction as per manufacturer's protocol (Sigma, St. Louis, MO). To obtain proliferating granulosa cells for *in vitro* culture, ovaries from 25-27 day old unprimed female CF-1 mice were collected into room temperature M-199 medium (Sigma) supplemented with 10 mM Hepes (Gibco, Carlsbad, CA) and 0.2% bovine serum albumin (Sigma). Ovaries were then transferred to M-199 media containing 1.8 mM EGTA and 0.5 M sucrose (Fluka, St. Louis, MO) and incubated for 15 minutes at 37°C. Following incubation, ovaries were rinsed three times in M-199 media to remove sucrose then were punctured as previously described. Expressed mural granulosa cells were centrifuged for 5 minutes, resuspended in Dulbecco Modified Eagle medium/ Ham F-12 (DMEM/F-12; Cellgro, Manassas, VA) supplemented with 10% heat

inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1% Gentamicin (Gibco). All procedures involving animals were reviewed and approved by the University Committee on the Use and Care of Animals at the University of Kansas Medical Center and performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals. All experiments were performed using CF-1 female mice from Charles River Laboratories, Wilmington, MA.

#### *Microarray and Temporal Expression Analysis*

Total RNA was quantified using a NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and quality was assessed by analysis of the 18S and 28S peaks on an Agilent 2001 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Three independent replicates of mural granulosa cells were collected before hCG (0 hour) and 4 hours post-hCG for detection of mature miRNA expression by microarray analysis. The 0 hour granulosa cell samples were labeled with Cy3 and the 4-hour hCG samples with Cy5. Each 0 and 4-hour replicate was then hybridized as a pair onto an individual Atactic  $\mu$ ParaFlo microfluidics chip (Sanger versions 8.2 and 9.0 [101, 102]; LC Sciences, Houston, TX) as described in detail by the manufacturer. These chips (n=3) included 357 experimentally validated miRNAs on the 8.2 Sanger chip and 375 on the version 9.0 chip. An additional 100 miRNAs predicted in human but not detected/represented in

the murine miRNA 8.2/9.0 Sanger databases were also printed onto the microarray chips to evaluate their expression in periovulatory granulosa cells (list provided upon request). This data was normalized using a cyclic LOWESS (Locally-weighted Regression) method as described by LC Sciences, Inc, to remove system related variations such as sample amount variations, dye labeling bias, and signal gain differences between scanners. Those signals that were greater than background plus three times the standard deviation were then derived for each color channel; the mean and the co-variance ( $CV = \text{st. dev.} \times 100 / \text{replicate mean}$ ) was calculated for each probe having a detected signal. A log transformed ratio was then calculated from the two sets of detected signals, and the transcript ratios with p-values of less than 0.01 were considered to be differentially expressed. For clustering analysis of multiple datasets, data adjustment included data filtering, Log2 transformation, and gene centering and normalization. Data filtering was also done to remove clustering values from the data set (detected signals or detected ratios below a threshold value). An in-depth cluster analysis was then completed using the paired samples, and differentially expressed transcripts were compiled ( $p\text{-value} < 0.05$ ). Lists of miRNAs showing their relative fluorescence values were generated after exclusion of all transcripts that failed to be present in two of three 0 hour and two of three 4 hour samples and those having fluorescence values of less than 50, which is approximately half of the background level. Additionally, the normalized and



background subtracted data was analyzed using the National Institute of Aging Array Analysis (<http://lgsun.grc.nia.nih.gov/anova/>) with significant genes identified as having  $p$ -values  $< 0.05$  [103].

#### *Quantitative RT-PCR for Mature miRNAs*

Quantitative RT-PCR (qRT-PCR) was performed to validate the microarray results and to analyze the temporal expression of specific mature miRNAs. Human miRNA assay kits for specific miRNAs (mmu-miR-132, mmu-miR-212, mmu-miR-21, and mmu-miR-31 hereafter referred to *Mirn132* and *Mirn212*, etc.) were purchased from Applied Biosystems (Foster City, CA) as were the reverse transcription kits. Total RNA was diluted to a 50 ng/ul working dilution then 250 ng were used in the RT reactions following the manufacturer's protocol with the following modifications. Briefly, 3 to 5 miRNAs were reverse transcribed in a single RT reaction using 2 ul of each miRNA specific 5x RT primer. This single RT reaction for multiple miRNAs then served as the starting material for independent qRT-PCR reactions using the primers and probes specific to each miRNA. In addition to the added benefit that only one fifth of the reverse transcription reagents and RNA were needed, a single reaction allowed the normalizer and target amplicons to be determined from the same RT reaction. Comparison of the qRT-PCR results for miRNAs that were reverse transcribed as a group versus those that were transcribed individually showed no differences, indicating that the RT primers

and the derived cDNAs do not interfere with the quantification of the other miRNAs. To normalize for starting material, a 20 ul aliquot from each of the 50 ng/ul total RNA sample dilutions was reverse transcribed using random hexamers and GAPDH levels determined using the Rodent GAPDH Taqman primers and probe (Applied Biosystems). Additionally, a reverse snRNA U6 RT/PCR primer (see **Table 2-1** for sequence) was included in some of the miRNA RT reactions and qRT-PCR of U6 (**Table 2-1**) following either miRNA RT or random hexamer RT demonstrated that GAPDH and U6 were equally valid for normalization (results not shown). Comparison of the qRT-PCR results using GAPDH, U6 snRNA or miR31 (miR31 was shown not to change in granulosa cells following *in vivo* LH treatment) as the normalizer made no difference in the relative profiles of the target miRNAs. As most of the initial *in vivo* studies used GAPDH, we chose to depict all of the data normalized to GAPDH levels for consistency throughout the paper. The qRT-PCR reactions were completed on the 7900 HT Sequence Detection System (Applied Biosystems). Each sample was run in triplicate, and the average used in subsequent calculations. Each primer set included a minus RT control. The delta-delta Ct method was used to calculate relative fold-change values between samples [104], and at least three independent replicate experiments were used to calculate means and SEM values.

#### *Quantitative RT-PCR for primary-miRNAs and Messenger RNAs*

To confirm that a single pri-miRNA exists for *Mirn132* and *Mirn212* and that it is transcriptionally upregulated in response to LH, a qRT-PCR primer set was designed to amplify a region that spans from the 5' end of the *Mirn212\_pre* through the 3' end of the *Mirn132\_pre*. These primers to the primary *Mirn132/212* (*Mirn132/212\_pri*; also known as pri-miR-132/212) transcript (see **Table 2-1** for primer sequences) amplify a 349 base pair amplicon that was confirmed by sequencing (data not shown).

Intron spanning primers for *Drosha*, *DGCR8*, *Exportin-5*, *Dicer1*, Methyl CpG binding protein (*MeCP2*), C-terminal binding protein 1 (*CtBP1*) and p250 GTPase activating-protein (*GAP*) (also known as *Grit* within the murine genome) were designed using the Primer Express 3.0 software (Applied Biosystems), and qRT-PCR was conducted as previously described (primer sequences provided in **Table 2-1**)

**TABLE 2-1:** *Primer sequences for reverse transcription and qRT-PCR.*

<b>Gene Name</b>	<b>Primer Sequence (5' -&gt;3')</b>
snRNA U6 RT-PCR	r: aacgcttcacgaatttcgt
snRNA U6 qRT-PCR	f: ctcgcttcggcagcaca r: aacgcttcacgaatttcgt
<i>Pri-Mirn132/212</i>	f: cggcaccttggtcttagact r: gggcgaccatggctgtag
<i>Grit</i>	f: ttgaagtgtcccaggttctt r: tatatcccatccacaatgccatac
<i>CtBP1</i>	f: ggcagcgggttgacaata r: gcacactgcgatgcctagatc
<i>MeCP2</i>	f: ttgatcaatccccaggga r: cattaggtccaaggaggtgtct
<i>Rnasen</i>	f: gaagtcaccgtggagctgagta r: atcattgcatgctgacagacatc
<i>DGCR8</i>	f: tcaagggtccgccctgtttat r: gaggcacccaaaaggctcactt
<i>Xpo5</i>	f: gacgcagaacatggaaagaatct r: tgtcttcatttgttgactgtttaca
<i>Dicer1</i>	f: cagctctggaccataacacaattg r: aggtcgcccctgatctgat

### *Granulosa Cell Culture*

Ovarian granulosa cells expressed from 25-27 day old mouse ovaries as previously described were seeded at  $2.5 \times 10^4$  cells per well into 6-well tissue culture plates (Corning) for temporal miRNA expression analysis and for anti-miRNA/locked nucleic acid (LNA) analysis or at  $1 \times 10^6$  cells per 10 cm tissue culture dishes (Corning) for Western analysis. All tissue culture plastics were pre-coated with fibronectin (2.5ul/ml) for 30 minutes at 37°C prior to plating cells. Cells were cultured at 37°C in an incubator supplemented with 5% CO<sub>2</sub>, and media was replaced 24 hours after plating to remove any unattached cells. At 48 hours post-plating, the cells were placed in serum-free DMEM/F-12 media for 24 hours at which point they were treated with 1 mM 8-bromo-cAMP (Sigma) for 1, 2, 4, 6, 8 or 12 hours or remained untreated (serum-free media) for the same time periods. Total RNA was isolated, and expression of *Mirn132* and *Mirn212* were analyzed using qRT-PCR.

### *Transfection*

Transfection of the mural granulosa cells with anti-microRNAs (anti-miRs) and LNA oligoribonucleotides to *Mirn132* and *Mirn212* was completed with Lipofectamine 2000 (Gibco) as described in the manufacturer's instructions. Briefly, isolated mural granulosa cells were grown for 48 hours prior to transfection, rinsed twice with serum-free DMEM/F12 and transfected

with a non-specific anti-miR negative control (42nM) or with hsa-miR-132 anti-miR (21nM) and hsa-miR-212 anti-miR (21nM; Ambion) individually or in combination. The anti-miR to *Mirn212* failed to consistently block the *Mirn212* action and/or detection of the mature miRNA transcript. Therefore, we designed a set of LNA knockdown probes complementary to *Mirn132* (tggctgtagactgtta, underlined bases denote the LNA nucleotides) and *Mirn212* (tgactggagactgtta). Cells were transfected with non-specific LNA-scramble (40nM, IDT) or with a combination of *Mirn132*-LNA (20nM) and *Mirn212*-LNA (20nM). Transfection efficiency within this cell type was analyzed using a fluorescein-labeled BLOCK-iT Fluorescent Oligo (Invitrogen) transfected at 100nM into cultured granulosa cells using the same protocol as stated above, and the transfection efficiency was determined to be 70% using fluorescent microscopy. Following a 24-hour transfection, the cells were rinsed twice with serum-free DMEM/F-12 then treated with 1mM 8-bromo-cAMP for 24 hours. Total RNA was isolated and miRNA expression analyzed by qRT-PCR as previously described.

### *Western Blots*

Protein for Western blot analysis was collected from cAMP treated granulosa cells transfected with either the non-specific scramble LNA or with the combination of *Mirn132*-LNA and *Mirn212*-LNA then treated with 8-Br-cAMP for 24 hours. Following treatment, granulosa cells were collected in cell

lysis buffer (200 µl/10cm dish; Cell Signaling Technology, Danvers, MA), and protein levels were quantified using the Becton Dickinson Protein Assay (Hercules, CA). Standard Western blot protocols were used with anti-CtBP1 (1:1000, BD Biosciences) and anti-nucleoporin (1:500, BD Biosciences) antibodies diluted in TBST+5% BSA. Secondary HRP-conjugated antibodies were diluted in TBST+5% milk at 1:5000 for CtBP1 and nucleoporin detection. CtBP1/nucleoporin blots were exposed using pico ECL detection (Thermo Scientific).

### *Steroid Assays*

Concentrations of estradiol and progesterone in granulosa cell culture media were analyzed by radioimmunoassay as previously described [105]. Estradiol levels were quantified in one ml of media following ether extraction, while progesterone levels were measured following a 1:10 dilution of the medium in 0.1M PBS + 0.1% gelatin.

### *miRNA Bioinformatics*

To determine if mRNAs expressed within periovulatory granulosa cells might be regulated by LH-regulated miRNAs, a comprehensive list of *Mirn132* and *Mirn212* target transcripts was created using the PicTar algorithm [106]. We then compared this list of transcripts to two mRNA gene lists that included all genes that were marginal or present within murine granulosa cells at 0 and

1 hour post-hCG or at 8 hours post-hCG based on Affymetrix gene chip analysis. The resulting list of mRNA transcripts found to be predicted targets of *Mirn132* and *Mirn212* and to be expressed within granulosa cells was then compared to two additional miRNA target prediction algorithms (miRBase and miRanda) [46, 107]. A detailed description of the computational procedures used to compare these lists is available upon request.

### *Statistical Analysis*

Three independent microarray replicates were analyzed using an unpaired Student's t-test (LC Science). Statistical analysis of qRT-PCR data was performed with GraphPad Prism (version 4). All data were examined for heterogeneity of variance using Bartlett's test. If heterogeneity of variance existed, the data were log transformed and analyzed via one-way ANOVA. Upon determination of a significant ( $p < 0.05$ ) F-test, Newman-Keuls Multiple Comparison tests were used to determine differences among the means. P-values of less than 0.05 were considered significant.



## RESULTS

### *Identification of miRNAs Expressed within Mural Granulosa Cells*

Microarray analysis of miRNA expression in mural granulosa cells revealed that 196 and 206 detectable transcripts were present at 0 and 4 hours respectively in 2 of 3 samples (n=3). Each miRNA transcript was arbitrarily categorized into a group based on its fluorescence, a value indicative of its relative level within the cells. **Tables 2-2, 2-3** and **2-4** list the highly abundant (n=31, fluorescence ranging from 50,000 to 10,000), intermediate (n=64, fluorescence less than 10,000 to 1000) and low (n=117, fluorescence less than 1000 to 50) abundance miRNAs.

**TABLE 2-2:** High abundance microRNAs present in periovulatory granulosa cells isolated prior to and 4 hours after hCG stimulation.

High Abundance	MicroRNA Sequence	Relative Fluorescence	Chromosome	Old Sanger Nomenclature
mmu-let-7a-1	TGAGGTAGTAGGTTGTATAGT <b>T</b>	27015	13	mmu-let-7a
mmu-let-7b	TGAGGTAGTAGGTTGTGTGGTT	17747	15	
mmu-let-7c-1	TGAGGTAGTAGGTTGTATGGTT	22463	16	
mmu-let-7d	AGAGGTAGTAGGTTGCATAGT <b>T</b>	19368	13	mmu-let-7c
mmu-let-7e	TGAGGTAGGAGGTTGTATAGT <b>T</b>	11278	17	
mmu-let-7f-1	TGAGGTAGTAGATTGTATAGT <b>T</b>	20744	13	
mmu-let-7g	TGAGGTAGTAGTTGTACAGT <b>T</b>	12950	9	mmu-let-7f
mmu-let-7i	TGAGGTAGTAGTTGTGCTGT <b>T</b>	12316	10	
mmu-miR-125a-5p	TCCCTGAGACCCTTTAACCTGTGA <b>A</b>	15096	17	
mmu-miR-15b	TAGCAGCACATCATGGTTTACA	18059	3	mmu-miR-125a
mmu-miR-16	TAGCAGCACGTAATATTGGCG	31971	3,14	
mmu-miR-17	CAAAGTGCTTACAGTGCAGGTAG <b>(T)</b>	12162	14	
mmu-miR-195	TAGCAGCACAGAAATATTGGC	11705	11	mmu-miR-17-5p
mmu-miR-20a	TAAAGTGCTTATAGTGCAGGTAG	11415	14	
mmu-miR-21	TAGCTTATCAGACTGATGTTGA	10681	11	
mmu-miR-23a	ATCACATTGCCAGGGATTCC	11263	8	
mmu-miR-23b	ATCACATTGCCAGGGATTACC	14782	13	
mmu-miR-25	CATTGCACTTGTCTCGGTCTGA	13309	5	
mmu-miR-26a	TTCAAGTAATCCAGGATAGGCT <b>T</b>	21120	9,10	
mmu-miR-29a	TAGCACCATCTGAAATCGGTT <b>A</b>	10684	6	
mmu-miR-30b	TGTAACATCCTACACTCAGCT	13392	15	
mmu-miR-30c	TGTAACATCCTACACTCTCAGC	14194	1,4	mmu-miR-424
mmu-miR-322	CAGCAGCAATTCATGTTTTGGA	42177	X	
mmu-miR-322*	AAACATGAAGCGCTGCAACA <b>C</b>	11118	X	
mmu-miR-351	TCCCTGAGGAGCCCTTTGAGCCTG	12264	X	mmu-miR-322
mmu-miR-451	AAACCGTTACCATTACTGAGTT	51789	11	
mmu-miR-672	TGAGGTTGGTGTACTGTGTGTGA <b>A</b>	10155	X	
mmu-miR-689	CGTCCCGCTCGGCGGGGTCC	16527	1,16	
mmu-miR-709	GGAGGCAGAGGCAGGAGGA	24913	8	
mmu-miR-762	GGGGCTGGGGCCGGGACAGAGC	17370	7	
hsa-mir-638	AGGGATCGCGGGCGGGTGGCGGCCT	17131	19	

**TABLE 2-3:** Intermediate abundance microRNAs present in periovulatory granulosa cells isolated prior to and 4 hours after hCG stimulation.

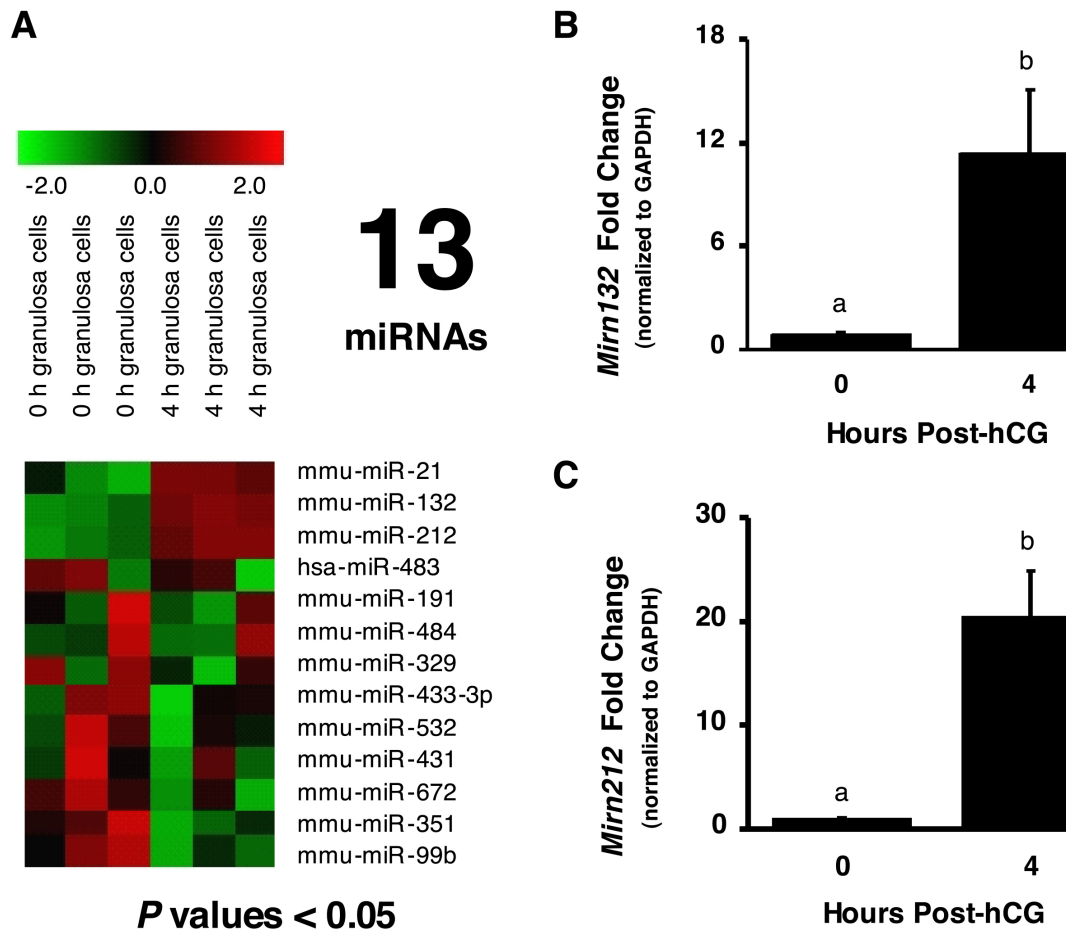
Intermediate Abundance	MicroRNA Sequence <sup>a</sup>	Relative Fluorescence	Chromosome	Old Sanger Nomenclature
mmu-let-7d*	CTATACGACCTGCTGCCTTTCT	1213	13	
mmu-miR-100	AACCCGTAGATCCGAACCTTG	1235	9	
mmu-miR-103	AGCAGCATTGTACAGGGCTATGA	6225	2,11	
mmu-miR-106a	CAAAGTGCTAACAGTGCAGGTAG	4237	X	
mmu-miR-106b	TAAAGTGCTGACAGTGCAGAT	5749	5	
mmu-miR-107	AGCAGCATTGTACAGGGCTATCA	5834	19	
mmu-miR-125b-5p	TCCCTGAGACCCCTAACTTGTA	7524	17	mmu-miR-125b
mmu-miR-126-3p	TCGTACCGTGAGTAATAATGCG	1521	2	
mmu-miR-127	TCGGATCCGTCTGAGCTTGGCT	3270	12	
mmu-miR-130a	CAGTGCAATGTTAAAAGGGCAT	2475	2	
mmu-miR-132	TAACAGTCTACAGCCATGGTCG	2333	11	
mmu-miR-140*	TACCACAGGGTAGAACACGG(A)	1152	8	
mmu-miR-143	TGAGATGAAGCACTGTAGCTC(A)	1215	18	
mmu-miR-145	GTCCAGTTTCCCAGGAATCCCT(T)	2408	18	
mmu-miR-152	TCAGTGCAATGACAGAACTTGG(G)	1330	11	
mmu-miR-15a	TAGCAGCACATAATGGTTGTG	4458	14	
mmu-miR-181a	AACATTCAACGCTGTGCGTGAGT	3595	1,2	
mmu-miR-181b	AACATTCAATGCTGTGCGTGGG	3084	1,2	
mmu-miR-185	TGGAGAGAAAGGCAGTTCCTGA	1524	16	
mmu-miR-191	CAACGGAATCCCAAAGCAGCTG	6507	9	
mmu-miR-199a-3p	(AT)ACAGTAGTCTGCACATTGGTTA	2253	1,9	mmu-miR-199a*
mmu-miR-19b	TGTGCAATCCATGCAGAACTGA	9035	X	
mmu-miR-202-3p	AGAGGTATAGCGCATGGGAAGA	2451	7	
mmu-miR-20b	CAAAGTGCTCATAGTGCAGGTAG	3609	X	
mmu-miR-214	ACAGCAGGCACAGACAGGCAAG(T)	2037	1	
mmu-miR-22	AAGCTGCCAGTTGAAGAACTGT	4868	11	
mmu-miR-24	TGGCTCAGTTCAAGCAGGAACAG	6702	8,13	
mmu-miR-26b	TTCAAGTAATCAGGATAGGT(T)	7749	1	
mmu-miR-27a	TTCAAGTGGCTAAGTTCCGC	2688	8	
mmu-miR-27b	TTCAAGTGGCTAAGTTCTGC	5004	13	
mmu-miR-290-5p	ACTCAAACTATGGGGGCATTT(TT)	1044	7	mmu-miR-290
mmu-miR-301a	CAGTGCAATAGTATTGTCAAAGC	1066	11	mmu-miR-301
mmu-miR-30a	TGTAACATCCTCGACTGGAAG	4912	1	mmu-miR-30a-5p
mmu-miR-30d	TGTAACATCCTCGACTGGAAG	4303	15	
mmu-miR-30e	TGTAACATCCTCGACTGGAAG (OV12)	1336	4	
mmu-miR-320	AAAAGCTGGGTTGAGAGGGCGA(A)	4514	14	
mmu-miR-341	TCGGTCGATCGGTGCGT(CAGT)	1048	12	
mmu-miR-361	TTATCAGAATCTCCAGGGGTAC	2076	X	
mmu-miR-379	TGGTAGACTATGGAACGTAGG	2520	12	
mmu-miR-434-3p	TTTGAACCATCACTGCATCCT	1595	12	
mmu-miR-450a-5p	(T)TTTTGGCATGTGTTCTTAATAT	6506	X,X	mmu-miR-450
mmu-miR-455	(AT)GCAGTCCAGGGCATATACAC(T)	1852	4	mmu-miR-455-3p
mmu-miR-484	TCAGGCTCAGTCCCTCCCGAT (OV14)	1049	16	
mmu-miR-486	TCCTGTACTGAGCTGCCCGAG	8230	8	
mmu-miR-494	TGAAACATACCGGAAACCTC	1009	12	
mmu-miR-503	TAGCAGCGGGAACAGTACTGCAG	6146	X	
mmu-miR-541	AAGGGATTCTGATGTTGGTCACACT	1358	12	
mmu-miR-671-5p	AGGAAGCCCTGGAGGGCTGGAG(G)	1850	5	mmu-miR-671
mmu-miR-680	GGGCATCTGCTGACATGGGG	1638	6,X,12	
mmu-miR-690	AAAGGCTAGGCTCACAAACCAA	9139	16	
mmu-miR-705	GGTGGGAGTGGGTGGGCA	6782	6	
mmu-miR-720	ATCTCGCTGGGGCCTCCA	1003	3	
mmu-miR-92a	TATTGCACTTGTCCCGCCTG (OV7)	7728	14,X	mmu-miR-92
mmu-miR-93	CAAAGTGCTGTTGCTGCAGGTAG	5870	5	
mmu-miR-98	TGAGGTAGTAAGTTGATTGTT	2207	X	
mmu-miR-99a	AACCCGTAGATCCGATCTTGTG	3216	16	
mmu-miR-99b	CACCCGTAGAACCGACCTTGGC	7812	17	
mmu-miR-744	TGCGGGCTAGGGCTAACAGC	1495	11	
mo-miR-151	TCGAGGAGCTCACAGTCTAGT(A) (OV8)	1919	7	mo-miR-151*
mo-miR-327	CCTTGAGGGGCATGAGGGT	1233		
mo-miR-352	AGAGTAGTAGTTGCATAGTA	3458	X	
hsa-mir-574-3p	CACGCTCATGCACACACCCACA	1254	4	hsa-mir-574
hsa-mir-663	AGCGGGGGCGCCGCGGACCGC	1999	20	
hsa-mir-92b	TATTGCACTCGTCCCGGCCTC	4667	1	AKA mmu-miR92b

**TABLE 2-4:** Low abundance microRNAs present in periovulatory granulosa cells isolated prior to and 4 h after hCG stimulation.

Low Abundance	miRNA sequence <i>(bold sequences are those miRNAs detected in whole ovarian tissues by Ho et al., 2007)</i>	Relative Fluorescence	Chromosome	Old Sanger Nomenclature
mmu-miR-101a	TACAGTACTGTGATACTGAAG <b>(G)</b>	435	4	
mmu-miR-101b	TACAGTACTGTGATAGTGAAG	685	19	
mmu-miR-10a	TACCGTGTAGACCGAAATTGTG	56	11	
mmu-miR-10b	<b>TACCGTGTAGACCGAAATTGTG (OV9)</b>	301	2	
mmu-miR-128-1	TACAGTAAACCGGTCTCTT <b>(T)</b>	365	1	mmu-miR-128a
mmu-miR-128-2	TACAGTAAACCGGTCTCTT <b>(C)</b>	299	9	mmu-miR-128b
mmu-miR-134	CAGTGAATGATGATGAAGGGGAT	799	16	
mmu-miR-134	TGTGACTGTGTGACAGAGGGG	626	12	
mmu-miR-144	TGAGTATGATGATGAT <b>(AG)</b>	379	11	
mmu-miR-146a	TGAGAACTGAATCGATGCGTT	78	11	mmu-miR-146
mmu-miR-146b	<b>TCATGCGCTACAGAACTTGT</b>	55	19	
mmu-miR-146b	TCATGCGCTACAGAACTTGT	579	15	
mmu-miR-149	TCTGGCTCGTGCTCTCACTCC	121	1	
mmu-miR-150	TCTCCAGCCCTGTGACAGTG	141	7	
mmu-miR-151-3p	CTAGACTGAGGCTCTTGAAG	465	15	
mmu-miR-154	TAGGTATCCGTGTTGCTTGG	363	12	
mmu-miR-155	TTATGCTGTAATGTGATAGGGT	50	16	
mmu-miR-177	ACTGAGTGTGAGGGCAGTGT <b>(AG)</b>	151	14	
mmu-miR-181c	<b>AACATTCACGTGTGTGAAT</b>	95	8	
mmu-miR-186	CAAGAAATTCCTCTTTGGGCTT	113	3	
mmu-miR-188-5p	CATCCCTTGATGTGTGAAGG <b>(T)</b>	60	X	mmu-miR-188
mmu-miR-188	<b>TAAAGTGCATCTGTGCGAGATAG</b>	626	14	mmu-miR-18
mmu-miR-194	<b>TGTAAAGACACATCCATGTGGA</b>	257	1,19	
mmu-miR-198a-5p	<b>CCCAGTGTTCAGACTACCTGTC</b>	488	1,9	
mmu-miR-198b <sup>7</sup>	CCCAGTGTTCAGACTACCTGTC	147	2	
mmu-miR-19a	TGTGCAATCTATGCAAACTGA	559	14	
mmu-miR-200a	<b>TACACTGTCTGTACCAATGT</b>	55	4	
mmu-miR-200b	<b>TATACTGTCTGTATATGA (G)</b>	174	4	
mmu-miR-200c	<b>TATACTGTCTGTATATGA (G)</b>	67	6	
mmu-miR-203	<b>GTTAAATGTTTAAAGCACTAG</b>	59	12	
mmu-miR-205	<b>TCTTTGATCCACCGAGTGTG</b>	78	7	
mmu-miR-210	CTGTGGTGTGACAGCGGCTGA	973	11	
mmu-miR-212	TAAACATCTCCAGTACAGGCCA	788	11	
mmu-miR-216b	<b>(G99)AATCTCTGACGCCAATGTGA</b>	114	11	
mmu-miR-217	TACTGCTACAGAACTGACTGG <b>(AT)</b>	111	11	
mmu-miR-221	<b>AGCTCAATGTGCTGGGTTTC</b>	769	X	
mmu-miR-222	AGCTCAATGTGCTGGGTTTC	626	X	
mmu-miR-223	TGTCAATGTTGCAATACGCCA	179	X	
Low Abundance	miRNA sequence <i>(bold sequences are those miRNAs detected in whole ovarian tissues by Ho et al., 2007)</i>	Relative Fluorescence	Chromosome	Old Sanger Nomenclature
mmu-miR-232-5p	AGCAAGCTCACAGCTATTGAG	400	16	
mmu-miR-232-5p	ACTCAAACTGGGGGCTTTTG	229	7	
mmu-miR-236-5p	AGGGCCGCCCTCAATCTGTT	128	2	mmu-miR-236
mmu-miR-236	GGGAGGAGGGCTGTTTCTCC	307	2	
mmu-miR-239 <sup>7</sup>	TGTTTACGCTCCACATAGAT	801	12	mmu-miR-239
mmu-miR-23c	<b>TAGCACCAATTGAATCGAGTT</b>	194	1,6	
mmu-miR-23c	<b>TAGCACCAATTGAATCGAGTTA</b>	129	1	
mmu-miR-300	TATCCAGGGCAGCTGTCTTC	116	12	
mmu-miR-30a <sup>7</sup>	CTTTTACGTGGATGTTTGACG	287	1	mmu-miR-30a-3p
mmu-miR-31	<b>AGGCAAGTGTGCGATAGCTG</b>	60	4	
mmu-miR-323-3p	<b>(G)CACATACAGCTGCACTCT</b>	50	12	mmu-miR-323
mmu-miR-324-3p	CCACTGCCAGAGTGTCTG <b>(GG)</b>	385	11	
mmu-miR-324-5p	CGCATCCCTAGGGGATGTTGT	243	11	
mmu-miR-328	CTGGCCCTCTCTGCGCTCGGT	404	8	
mmu-miR-329	<b>AACAGACCGAGCTAACCTTTT</b>	812	12	
mmu-miR-331-3p	<b>GCCCTGTGGCTATCTAGAA</b>	145	10	mmu-miR-331
mmu-miR-331-3p	TTCACTGCTATATGATGCT <b>(TT)</b>	335	12	mmu-miR-337
mmu-miR-339-5p	TCCCTCTCTCCAGAGCTCACG	67	5	
mmu-miR-342-3p	<b>TCTCACAGAGAAATGCCAGCTG (G)</b>	800	12	mmu-miR-339
mmu-miR-345-5p	<b>(T)GCTGACCCCTATGCCAGTCT</b>	109	12	mmu-miR-342
mmu-miR-346	TGTCGTCCCGAGTGTGCTGCT	162	14	mmu-miR-345
mmu-miR-346	TGGCAGTGTCTAGCTGGTTGT	354	4	
mmu-miR-34b-5p	<b>(T)AGCGAGTGAATAGCTAATGT</b>	82	9	mmu-miR-34b
mmu-miR-34c	AGGCACTGATAGTACGTATGC	331	9	
mmu-miR-350	TTCCAGAGGCCATACACTTCA	756	1	mmu-miR-352
mmu-miR-370	<b>GCCTGTGGGGTGAACTGTGAT</b>	77	X	
mmu-miR-374	<b>ATATATCAACTCTGAAGT (T)</b>	59	12	
mmu-miR-376	ATCATAGAGAGCTCACT <b>(A)</b>	876	X	
mmu-miR-377	ATCATAGAGAGCTCACT <b>(T)</b>	565	12	mmu-miR-374-5p
mmu-miR-378	<b>ACTGGACTTGAGTCAGAGG (CG)</b>	292	12	
mmu-miR-378	ACTGGACTTGAGTCAGAGG <b>(CG)</b>	392	18	mmu-miR-422b
mmu-miR-380-3p	TATATGATGTGTCACATCTT	53	12	
mmu-miR-381	<b>TATACAGGCGCAAGCTCTCT</b>	53	12	
mmu-miR-382	<b>GAAATGTGCTGGTGACCCCT (T)</b>	68	12	
mmu-miR-409-3p	<b>GAATGTGCTGGTGACCCCT (T)</b>	725	12	mmu-miR-409
mmu-miR-409-3p	<b>GAATGTGCTGGTGACCCCT (T)</b>	476	12	
mmu-miR-410	ATATACACAGATGGCTG <b>(T)</b>	54	12	
mmu-miR-431	TGTCCTGCAAGCGCTATCA <b>(GG)</b>	205	12	
mmu-miR-433	ATCATGATGGGCTCTGGTGT	608	12	mmu-miR-433-3p
mmu-miR-434-5p	<b>(A)GCTGCACTGATGCTTGAACCA</b>	289	12	
Low Abundance	miRNA sequence <i>(bold sequences are those miRNAs detected in whole ovarian tissues by Ho et al., 2007)</i>	Relative Fluorescence	Chromosome	Old Sanger Nomenclature
mmu-miR-455 <sup>7</sup>	ATTTGGGACAAATTGGATGAT	426	X	
mmu-miR-455-5p	TATGTGCGATTTGGATGATG	50	4	mmu-miR-455-5p
mmu-miR-468	<b>TATATACACACACATAGAG (G)</b>	423	2	
mmu-miR-467a	<b>TATATACACACACACATAGAG</b>	224	2	
mmu-miR-467	GTAAGTGTGCTGATATATG	99	2	
mmu-miR-467b	ATATACACACACACACACAC	50	2	
mmu-miR-465-5p	AGAGCTGGCGCTGATGAATC	60	12	
mmu-miR-465-5p	AGATACACGGCTCTCTCTC	224	12	
mmu-miR-467b	ATCGTACAGGGCTGCGAT <b>(T)</b>	231	12	
mmu-miR-485	AACAAAGATGCTGCTTGT	623	12	
mmu-miR-487	CAGCAGCACTGTGTTTGT	740	11	
mmu-miR-501 <sup>7</sup>	ANTGCAACCGGCAAGGATTTG	97	X	mmu-miR-501 <sup>7</sup>
mmu-miR-502	CATGCTTGATGTGAGACGCT	539	X	
mmu-miR-540	AGTCAAGCTGATCTCTGG	50	12	
mmu-miR-545-3p	TGTGACAGATGATAGTGA	701	X	
mmu-miR-545-3p	CTGGGGATCATCATGTACGA	118	X	
mmu-miR-543	AAAGATCGGGGTGACGCT <b>(T)</b>	325	12	
mmu-miR-615-3p	TGGAGGCTGGCTCCGCTGT	78	15	
mmu-miR-652	ANTGCGCGCACTAGGCTGTGGA	870	X	
mmu-miR-665	ACCAGAGGCTGAGTCT <b>(TA)</b>	75	12	
mmu-miR-667	TGCACCTGGCAGCCAGCCAG	236	12	
mmu-miR-668	TGTCACTGGCTGGCCCACTAGC	50	12	
mmu-miR-668a	AGTTGTGTGATGTTCACT	201	2	
mmu-miR-669b	ATGTTGTGTGTGGAATGTTGT	287	2	
mmu-miR-674	GCAGTGAGATGGGAGTGTGTA	542	2	
mmu-miR-674 <sup>7</sup>	CACAGCTCCGATCTCAGAACAA	277	2	
mmu-miR-676	CCGTCTGAAGTGTGTTGCT	187	X	
mmu-miR-7a	TGGAAGACTGATGTTTGTGT	100	7,13	
mmu-miR-714	CQACAGGCGCGCTGGGTGGC	353	Unknown	
mmu-miR-714	<b>(GAATTT)GGGCTGTGGCTCTGTGGGA (G)</b>	330	3	
mmu-miR-685	GAATATCATGGACTAGGG	627	MT	
mmu-miR-192	CTGACCTATGATTGACAGCC	140	1	
mmu-miR-221 <sup>7</sup>	<b>AATTTCTGATGGCAAGCTT (OV19)</b>	162	10	
mmu-miR-409-3p	ANTGTGTGGTGAACCG	322	6	
mmu-miR-411	TAGTAGACCTATAGCGTAGC	465	14	
mmu-miR-421	ATCAACAGCAATATTTGGGCGC	667	X	
mmu-miR-433-5p	AGAGCAGAGAGAGAGAGAG	472	11	
mmu-miR-431	TGCTGTAGATGTTGTGAGAG	113	9	
mmu-miR-412	GCTGGGAGGGGCTGTGAGCTCTT	93	11	
mmu-miR-437	ACTGGGGGCTTTGGGCTCTGCT	50	19	

A pairwise t-test cluster analysis of the data from the microarrays found 13 miRNA transcripts to be differentially expressed between 0 and 4 hours post-hCG (p-values < 0.05) (**Figure 2-1A**). Analysis using the National Institute of Health Array Analysis tool also found *Mirn132* and *Mirn212* to be significantly upregulated between the 0 and 4 hour time points with fold changes of 16.9 and 21.7, respectively. Quantitative RT-PCR was used to validate the microarray results for these two specific LH-induced miRNAs, as well as one other highly upregulated miRNA transcript (*Mirn21*, data not shown). The fold increases seen for the mature forms of both *Mirn132* ( $11.4 \pm 4.2$ ) and *Mirn212* ( $20.5 \pm 4.0$ ) at 4 hours post-hCG (**Figures 2-1B** and **2-1C**) were similar to the expression changes predicted by miRNA microarray analysis.

Figure 2-1



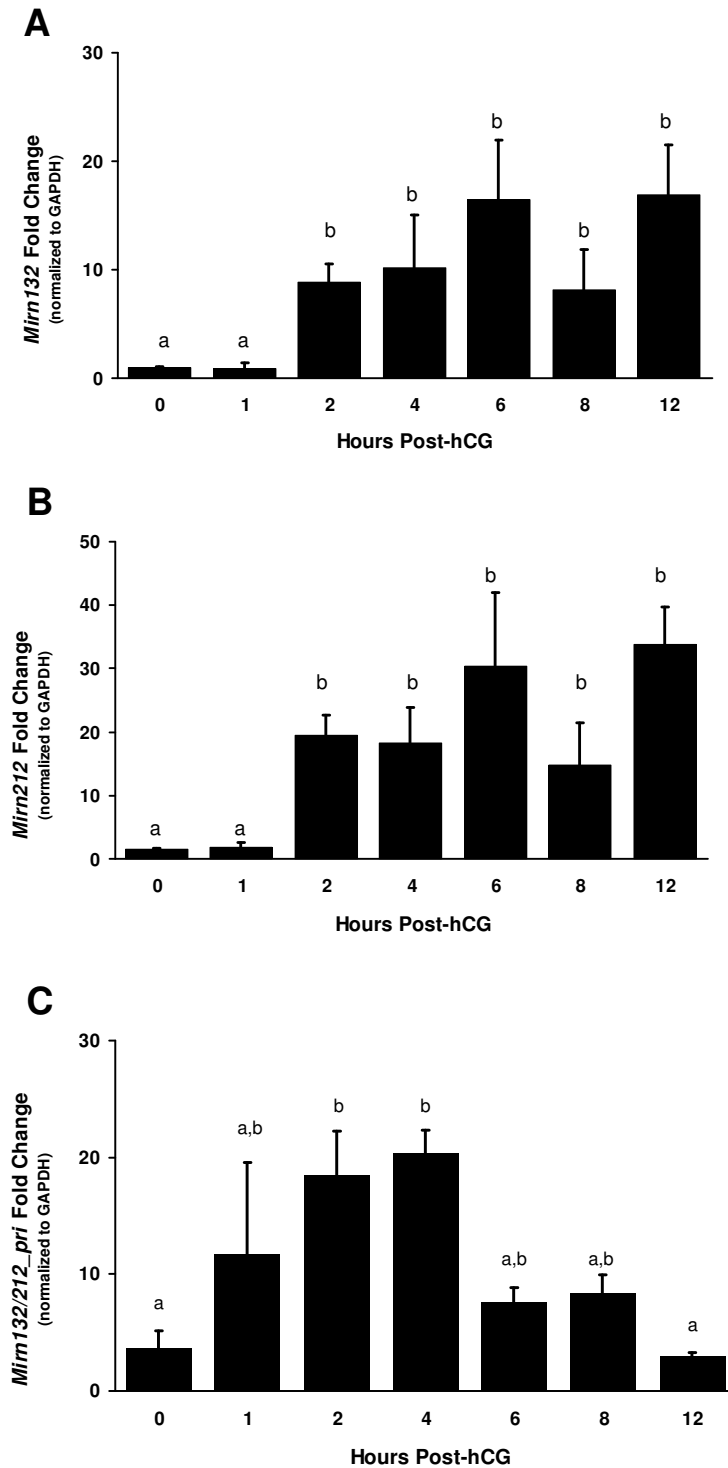
**FIGURE 2-1:** Microarray cluster analysis for mural granulosa cells collected 46 hours after *in vivo* eCG (0 hour control) or after eCG + 4 hours post-hCG treatment of mice. Paired t-test analyses of the microarray data (n=3) revealed: **(A)** 13 miRNA transcripts to be statistically different ( $P < 0.05$ )

### *Temporal Expression of miRNAs in Granulosa Cells In Vivo*

*In vivo* expression patterns for mature *Mirn132* and *Mirn212* were determined by qRT-PCR within mural granulosa cells. Examination of miRNA levels over the 12 hour periovulatory period demonstrated that *Mirn132* and *Mirn212* expression remained unchanged 1 hour following hCG administration, then were significantly upregulated between 2 hours and 12 hours post-hCG to 17- and 34-fold over the basal levels (0 hour control) for *Mirn132* and *Mirn212*, respectively (**Figure 2-2A** and **2-2B**).

Quantitative RT-PCR analysis of the pri-miRNA transcript in periovulatory granulosa cells collected after *in vivo* hCG treatment indicated that both the *Mirn132* and *Mirn212* originate from a single transcript and that increased expression occurred approximately 1 hour prior to the detection of the mature forms (**Figure 2-2C**). Changes in pri-miRNA expression preceded those changes that were observed in the mature forms (when comparing **2-2C** to **2-2A** and **2-2B**).

**Figure 2-2**

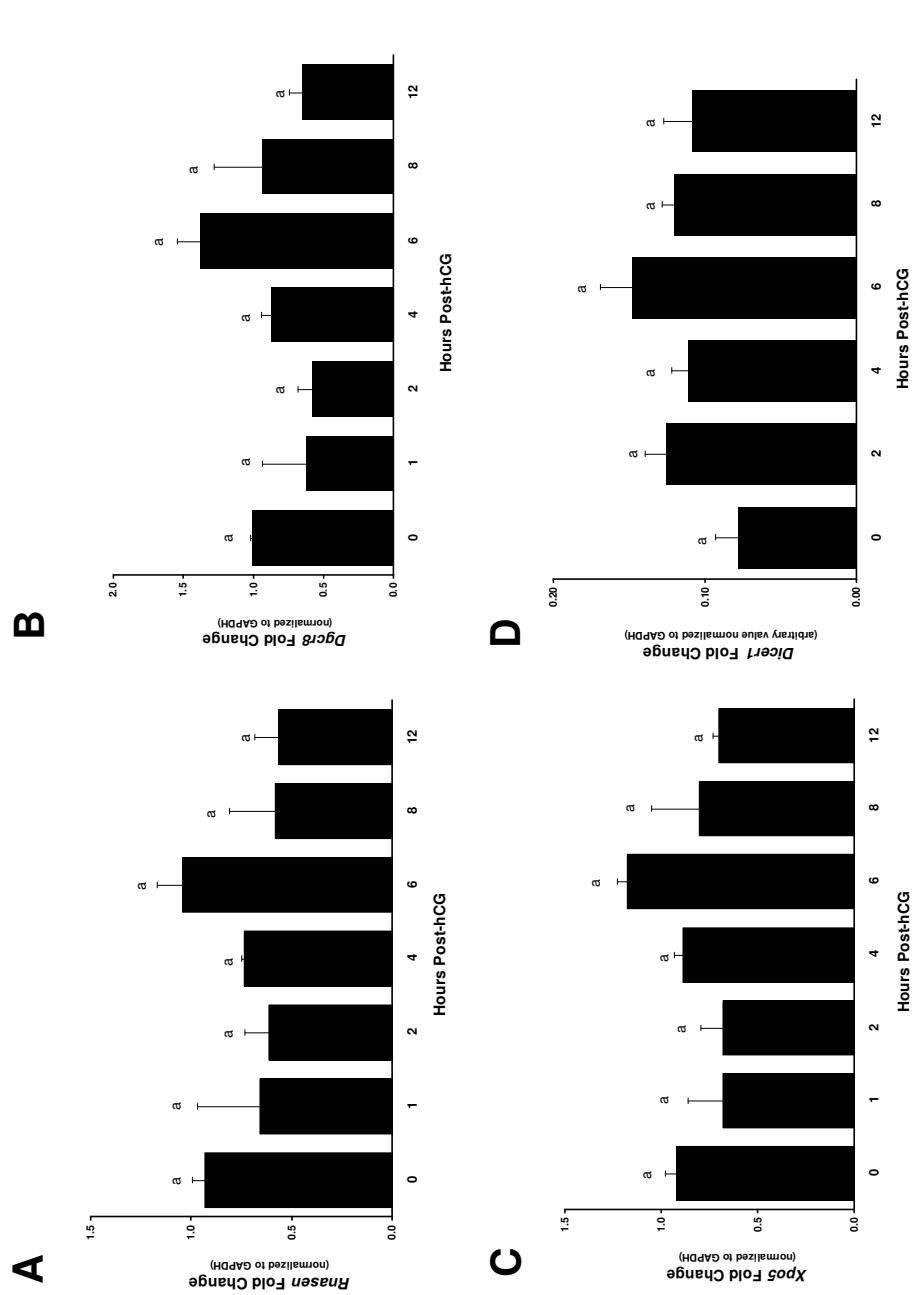


**FIGURE 2-2:** *qRT-PCR analysis of mature Mirn132 (A), mature Mirn212 (B) and the Mirn132/212\_pri transcript levels in mural granulosa cells collected from mice following in vivo treatment of eCG for 46 hours (i.e., 0 hour) and eCG + hCG for 1 to 12 hours post-hCG. Mature Mirn132 and Mirn212 levels were normalized to GAPDH. <sup>a,b</sup> Means  $\pm$  SEM with different superscripts are different ( $P < 0.05$ ).*



Quantitative RT-PCR analysis for the factors involved in the synthesis of miRNAs indicated the presence of the essential miRNA processing components (i.e., Rnasen (*Drosha*), *Xpo5*, *DGCR8* and *Dicer1*) within the purified population of granulosa cells and showed that an ovulatory surge of LH/hCG had no effect on the expression of these factors *in vivo* (see **Figure 2-3**).

Figure 2- 3

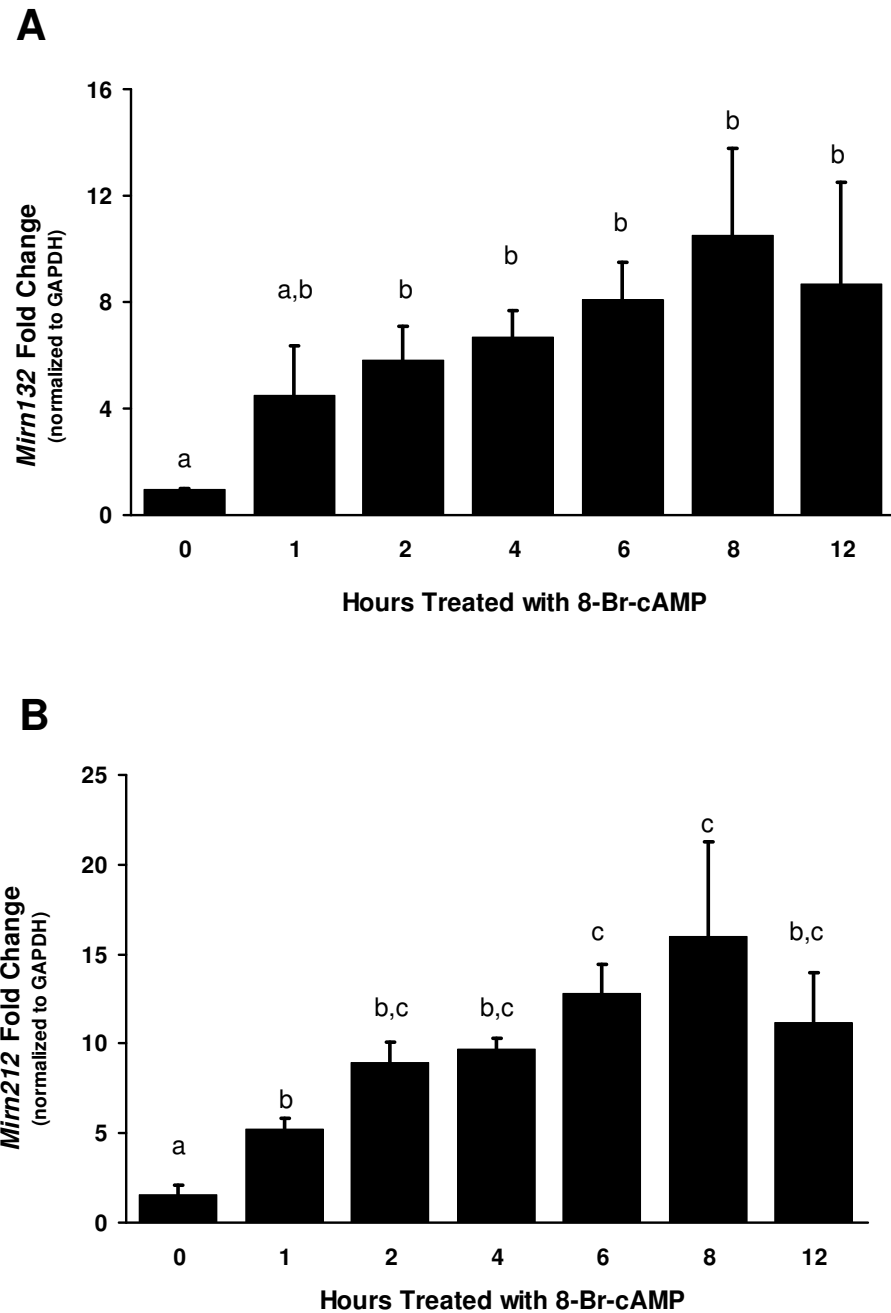


**FIGURE 2-3:** qRT-PCR analysis of the microRNA processing factors **(A)** *Rnaseh* (*Drosophila*) **(B)** *Dgcr8* **(C)** *Xpo5* and **(D)** *Dicer1* mRNA levels in mural granulosa cells collected from mice following in vivo treatment of eCG for 46 hours (i.e., 0 hour) and eCG + hCG for 1 to 12 hours post-hCG. All data was normalized to GAPDH. <sup>a</sup> Means  $\pm$  SEM were not different ( $P < 0.05$ ).

### *Temporal Expression of miRNAs in Granulosa Cells in Vitro*

To determine whether LH/hCG-induced miRNAs were downstream of the cAMP signal transduction pathway, isolated granulosa cells from preovulatory follicles (27d old mice) were placed in culture. Plates were typically ~20% confluent one day following plating. Cells continued proliferating in culture so that by the second day the cells were near 50% confluent. On the third day of culture, cells were exposed to 8-Br-cAMP treatment for 0 to 12 hours, mimicking the predominant second messenger system downstream of LH/hCG used in our *in vivo* experiments. The *in vitro* granulosa cell expression patterns for *Mirn132* and *Mirn212* were similar to that observed *in vivo*. However, the increases seen *in vitro* were more rapid as *Mirn132* and *Mirn212* were increased within 1 hour of 8-Br-cAMP treatment. Mature *Mirn132* and *Mirn212* levels remained elevated for the entire 12 hour cAMP treatment reaching their peak levels (10- and 16-fold increases respectively) at 8 hours post treatment (**Figures 2-4A and 2-4B**).

Figure 2-4

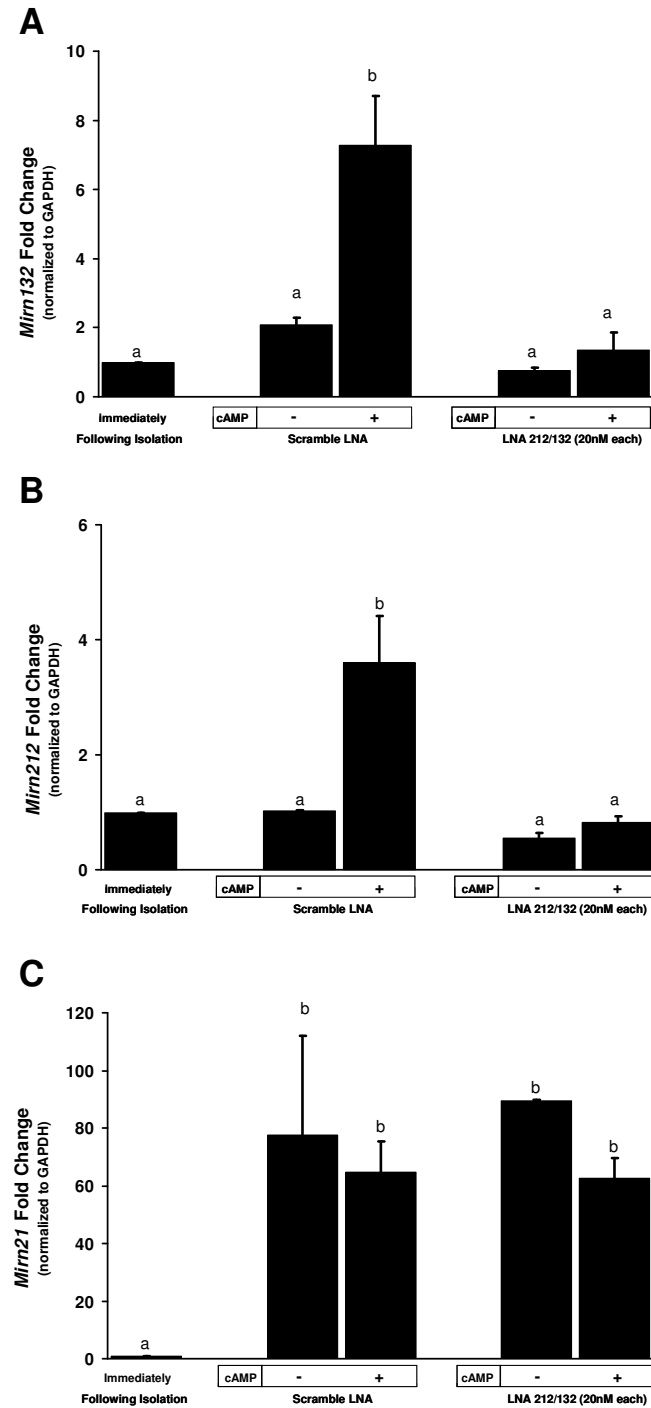


**FIGURE 2-4:** *qRT-PCR* analysis of mature *Mirn132* (**A**) and *Mirn212* (**B**) expression in cultured granulosa cells following treatment with 8-Br-cAMP. Mature *Mirn132* and *Mirn212* levels were normalized to GAPDH. <sup>a,b,c</sup> Means  $\pm$  SEM with different superscripts are different ( $P < 0.05$ ).

*Analysis of miRNA Expression Following In Vitro miRNA Knockdown with LNA Oligonucleotides*

To gain insight as to what *Mirn132* and *Mirn212* are regulating in granulosa cells and to begin to dissect the functional role these miRNAs have on granulosa cell function, we chose to knockdown expression of these factors using LNA oligonucleotides complementary to the miRNAs. Transfection combining LNA oligonucleotides for both *Mirn132* and *Mirn212* were effective in knocking down their respective target miRNAs (Figure 2-5). The cAMP stimulated levels of mature *Mirn132* (**panel A**) and *Mirn212* (**panel B**) were completely blocked by the LNA treatment. Conversely, treatment with the LNA oligos specific to *Mirn132* and *Mirn212* had no effect on the expression of *Mirn21* (**Figure 2-5C**), a miRNA that is unresponsive to cAMP stimulation *in vitro* but is upregulated within granulosa cells following the LH surge (data not shown).

Figure 2-5



**FIGURE 2-5:** *qRT-PCR analysis of mature Mirn132, Mirn212 and Mirn21 expression in cultured granulosa cells following transfection with LNAs to Mirn132 and Mirn212 and subsequent treatment with 8-Br-cAMP (n=3). Granulosa cells were transfected with Mirn132-LNA and Mirn212-LNA (20nM of each) as well as a non-specific scramble LNA (40 nM) for 24 hours prior to treatment with 8-Br-cAMP. Cells were then collected 24 hours following 8-Br-cAMP treatment. Mature levels of Mirn132 (A), Mirn212 (B) and Mirn21 (as known as miR-21) (C) were normalized to GAPDH. <sup>a,b</sup> Means  $\pm$  SEM with different superscripts are different ( $P < 0.05$ ).*

Following knockdown with a combination of 132 and 212 LNA oligonucleotides, we evaluated whether there was any effect on steroidogenesis. Medium concentrations of estrogen and progesterone were measured at the completion of the each experiment and knockdown of the miRNAs failed to affect medium estradiol or progesterone at 12 and 24 hours after cAMP treatment. Treatment of granulosa cells with cAMP increased progesterone synthesis ~ 6-fold as expected (steroid data not shown).

#### *Identification of Mirn132 and Mirn212 Targets*

To gain insight into the possible target mRNAs that these miRNAs may regulate, a comprehensive list of *Mirn132* and *Mirn212* target genes was created through the use of the PicTar algorithm [106]. This analysis yielded 156 or 157 mRNA transcripts as predicted targets of murine *Mirn132* and *Mirn212*, respectively. A comparison of these transcripts to genes expressed in granulosa cells at either 0 and 1 hour post-hCG or 8 hours post-hCG revealed 77 common transcripts (see **Table 2-5**). These transcripts were then evaluated using several other miRNA target prediction algorithms, and those results are also shown in Table 2-5. Ingenuity pathway analysis indicated that a large number of these transcripts were in the cellular development and gene expression ontology groups.

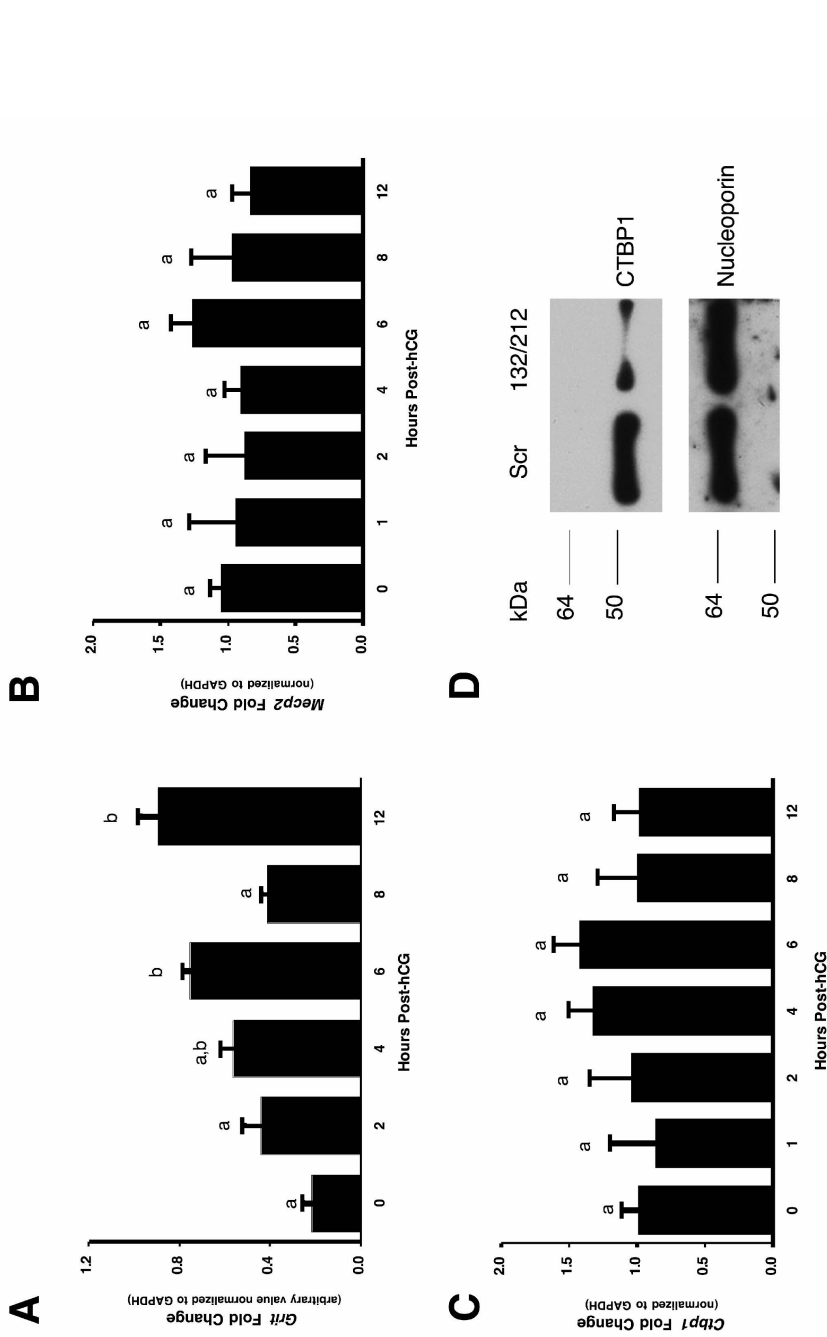
**TABLE 2-5: Ovarian granulosa cell mRNA transcripts that contain putative *Mirn132* recognition sites.**

GENE NAME	Ref Seq DNA ID	Microarray*	miRanda and miRBase Algorithm Comparison <sup>#</sup>
281048505Rik, RIKEN cDNA 281048505 gene (281048505Rik), mRNA	NM_176836	A, B	1
4732481H14Rik, RIKEN cDNA 4732481H14 gene (4732481H14Rik), mRNA	NM_172468	A	
5730555F13Rik, modulator of estrogen induced transcription isoform a	NM_025690	A, B	1, 2
5830417C01Rik, UPF0326 protein CGI-146 homolog	NM_024282	A	
6330407G11Rik, RIKEN cDNA 6330407G11 gene (6330407G11Rik), mRNA	NM_023423	A, B	1
6720463E02Rik, Dynein light chain 2, cytoplasmic (8 kDa dynein light chain) (DLC8) (DLC8b).	NM_026556	A, B	1, 2
Aebp2, AE binding protein 2	NM_178803	A, B	1
Arid2, AT rich interactive domain 2 (Arid-rfx like)	NM_175251	A, B	
Atf2, activating transcription factor 2	NM_009715	A, B	1
AU024582, expressed sequence AU024582	NM_153125	A, B	
Bicd2, bicaudal D homolog 2 (Drosophila)	NM_029791	A	
Binp2, BCL2/adenovirus E1B 19kDa-interacting protein 1, NIP2	NM_016787	A, B	1, 2
Brcal, breast cancer 1	NM_009764	A, B	1
Calu, calumenin	NM_007594	A, B	1, 2b
Cil2, cofilin 2, muscle	NM_007688	A, B	1
Cnif, cornichon homolog (Drosophila)	NM_009919	A, B	1
CSDIE1_MOUSE, Cold shock domain protein E1.	NM_144901	A, B	1
D15Er0621e, MKIAA0493 protein (Fragment).	NM_145959	A, B	1
Dnmt3a, DNA methyltransferase 3A	NM_153743	A	1
Edn1, endothelin 1	NM_010104	B	
Ef4a2, eukaryotic translation initiation factor 4A2	NM_013506	A, B	1, 2
Fkbp2, FK506 binding protein 2	NM_008020	A, B	2
Fubp1, far upstream element (FUSE) binding protein 1	NM_057172	A, B	1
Gpiap1, GPI-anchored membrane protein 1	NM_016739	A, B	1
Gsk3b, glycogen synthase kinase 3 beta	NM_019827	A	
H2afz, H2A histone family, member Z	NM_016750	A, B	1
H3f3a, H3 histone, family 3B	NM_008211	A, B	1
Hbegf, heparin-binding EGF-like growth factor	NM_010415	A	1, 2
Hn1, hematological and neurological expressed sequence 1	NM_008258	A, B	1, 2
Hnrph1, heterogeneous nuclear ribonucleoprotein H1	NM_021510	A, B	
Hnrpm, heterogeneous nuclear ribonucleoprotein M	NM_029804	A, B	1, 2
Iiga9, integrin alpha 9	NM_133721	A	
Lass2, longevity assurance homolog 2 (S. cerevisiae)	NM_029789	A, B	
Map3k12, mitogen activated protein kinase kinase kinase 12	NM_009582	A	1
Mapk1, mitogen activated protein kinase 1	NM_011949	A, B	
Mecp2, methyl CpG binding protein 2	NM_010788	A, B	1
MYST histone acetyltransferase 2	NM_177619	A, B	
Nfya, nuclear transcription factor-Y alpha	NM_010913	A, B	1
Nlk, nemo like kinase	NM_008702	A, B	
Olfm1, olfactomedin 1	NM_019498	A	1
Osbpl11, oxysterol binding protein-like 11	NM_176840	A, B	
Paip2, polyadenylate-binding protein-interacting protein 2	NM_026420	A, B	1, 2
Pde7b, phosphodiesterase 7B	NM_013875	A	1b
Pln2, profilin 2	NM_019410	A, B	1, 2b
Plagl2, pleiomorphic adenoma gene-like 2	NM_018807	A	1
Pnn, pinn	NM_008891	A, B	1, 2
Pom121, nuclear pore membrane protein 121	NM_148932	A, B	1
Ppm1g, protein phosphatase 1G (formerly 2C), magnesium-dependent, gamma isoform	NM_008014	A, B	1, 2
Prkm, protein kinase C, mu	NM_008858	A	1a, 2a
Pten, phosphatase and tensin homolog	NM_008960	A, B	1
Qk, quaking	NM_021881	A, B	
Rtn4, reticulon 4	NM_194051	A, B	
Sema4g, sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4G	NM_011976	A	1
Sfrs1, splicing factor, arginine/serine-rich 1 (ASF/SF2)	NM_173374	A, B	
Sirt1, sirtuin 1 ((silent mating type information regulation 2, homolog) 1 (S. cerevisiae)	NM_019812	A	1
Slc30a6, solute carrier family 30 (zinc transporter), member 6	NM_144798	A	1
Smarca5, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5	NM_053124	A, B	
Snag1, sorting nexin associated golgi protein 1	NM_130796	A	1a
Sox5, SRY-box containing gene 5	NM_011444	A	1
Tcf3, transcription factor 3	NM_009332	A, B	1, 2
Tcf7l2, transcription factor 7-like 2, T-cell specific, HMG-box	NM_009333	A	1a
TIM9A_MOUSE, Mitochondrial import inner membrane translocase subunit TIM9 A.	NM_013896	B	1, 2
Tloc1, translocation protein 1	NM_027016	A, B	1
Tmeff1, transmembrane protein with EGF-like and two follistatin-like domains 1	NM_021436	B	1, 2a
Tmem49, transmembrane protein 49	NM_029478	A, B	1a
Trib2, tribbles homolog 2 (Drosophila)	NM_144551	A	1a
Trim2, tripartite motif protein 2	NM_030706	A	1
Tsc22d3, TSC22 domain family 3	NM_010286	A	1
Ube2d3, ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast) mRNA	NM_025356	A, B	1a
Usp9x, ubiquitin specific peptidase 9, X chromosome	NM_009481	A, B	
Vapa, vesicle-associated membrane protein, associated protein A	NM_013933	A, B	
Vdac2, voltage-dependent anion channel 2	NM_011695	A, B	
Wdr42a, WD repeat domain 42A	NM_153555	A, B	
Zab2, zinc finger E-box binding homeobox 2	NM_001039180	A	
Zfx1b, zinc finger homeobox 1b	NM_015753	A	1
Zfp238, zinc finger protein 238	NM_001012330	A	1, 2a
Zfx, zinc finger protein X-linked	NM_011768	A, B	1



Additionally, three recently identified *Mirn132* target transcripts were examined to determine if they were differentially expressed in murine granulosa cells. Quantitative RT-PCR indicated that LH increased expression of *Grit* (the mouse homolog to p250 GAP) at 6 and 12 h post-hCG (**Figure 2-6A**). Conversely, *MeCP2* and *CtBP1* were not transcriptionally regulated (**Figure 2-6B** and **2-6C**). Western blot analysis for CTBP1 protein in granulosa cells following *in vitro* LNA treatment indicated that loss of miRNA function caused a marked decline in the translation of this target protein (**Figure 2-6D**).

Figure 2-6



**FIGURE 2-6:** qRT-PCR and Western blot analysis of several *Mirn132/212* target transcripts in granulosa cells collected from mice throughout the periovulatory period. **(A,B,C)** *CtBP1*, *MeCP2* and *Grit* mRNA levels were normalized to GAPDH. <sup>a, b</sup> Means  $\pm$  SEM with different superscripts are different ( $P < 0.05$ ). **(D)** CTBP1 protein levels in granulosa cells treated with a scrambled LNA or oligonucleotides complementary to *Mirn132/212*. Nucleoporin levels were used to confirm equal loading of the nuclear extracts. Similar results were obtained in six different replicates.

## DISCUSSION

This study is the first to describe the hormonal regulation of miRNA expression in the ovary, specifically the ability of the LH/hCG surge to mediate changes in the expression of miRNAs within periovulatory granulosa cells. The ovarian granulosa cell is likely a prime target for miRNA mediated changes in gene expression due to the rapid conversion this cell must undergo following the LH surge. This phenotypic change requires the global loss of its follicular phenotype and its attendant gene expression/protein profile as well as the rapid induction of a luteal phenotype. We chose to examine the expression and function of the two miRNAs (*Mirn132* and *Mirn212*) that exhibited the greatest difference in expression following the hCG/LH surge. The recent functional description of *Mirn132* in neuronal cells, combined with the fact that both *Mirn132* and *Mirn212* are regulated by the cAMP regulatory element binding protein (CREB), also made these miRNAs attractive to study [61].

From the microarray data, we identified 212 independent, mature miRNA transcripts in periovulatory mural granulosa cells that were above background levels in at least 2 of 3 samples for the 0 and 4 hour time points. These results are in striking contrast to the list of 122 miRNAs cloned from whole ovarian tissues recently by Ro et al. [108]. Of interest, approximately 20% of these transcripts are variants at the 5' and 3' ends, and as noted by the authors, a consequence that may be due to the cloning procedure.

Alternatively, editing of the miRNA ends, which was recently described, may also be involved [109]. A more conservative interpretation indicates that they identified 97 miRNAs combined from two week-old and adult mouse ovaries. Comparison of our list to the Ro et al. [108] list of 97 bonafide miRNAs cloned from whole ovaries indicates that ~80% of the transcripts overlap. Moreover, the relative abundance of each miRNA transcript (i.e., fluorescent value) observed in our experiments was predictive of the cloning method's ability to detect the transcript in the whole ovary. For example, 24 of 31 miRNA transcripts in our high fluorescence group, 28 of 64 miRNA transcripts in our intermediate, and 25 of 117 miRNA transcripts in the low group were detected by Ro et al. (2007) [108]. Those transcripts found in both lists are bolded in Tables 2-2, 2-3, and 2-4. Interestingly, several miRNAs including *Mirn132* and *Mirn212*, the two differentially expressed miRNAs with intermediate to low fluorescent levels we detected, were not detected in their analysis at all. Because our preparations of granulosa cells are not contaminated with oocytes as they were eliminated by size exclusion filtering, we can be confident that our results reflect the changes seen in the somatic cell compartments and are not the result of an effect of the germ cells/oocytes. Therefore, we believe that the miRNA microarray approach we used has provided us with a robust analysis of miRNA expression within the periovulatory granulosa cell. We recognize that novel granulosa cell miRNAs

may have been missed in our approach; however, presently this approach yields a much higher number of miRNAs as compared to a cloning method.

The miRNAs that fail to experience a differential expression pattern within granulosa cells after the LH surge have the potential to be very interesting as they may also play a role in ovarian function. Indeed, it has recently been demonstrated that miRNAs can both block and/or enhance translation dependent upon the cell cycle state [97]. MiRNAs regulate translation by recruiting the RNA induced silencing complex to the 3'UTR of mRNAs where they can prohibit further translation [96]. Recently, Vasudevan et al. [30] demonstrated that miRNAs could enhance translation under conditions that promote cellular differentiation by recruiting Argonaute 2 to this complex. Under this paradigm, highly expressed miRNAs in particular, while not exhibiting hormonally dependent regulation in expression, may still exhibit a dynamic regulatory role in response to a hormone-dependent shift of a key regulatory factor (i.e., an argonaute protein). This type of cellular event occurs in periovulatory granulosa cells as they transition from highly proliferative follicular cells to terminally differentiated luteal cells and could indicate that these highly abundant ovarian granulosa cell miRNAs, which are present prior to the LH surge, may be preventing the translation of genes critical for luteinization and ovulation. It is possible that these miRNAs only release their block on mRNA translation following the LH surge; alternatively these miRNAs may be necessary for the efficient translation of specific

mRNAs in the ovary after the LH surge. Subsequent studies will be necessary to determine the importance specific miRNAs have on ovarian granulosa cell function.

The qRT-PCR results for *Mirn132* and *Mirn212* indicated that *in vivo* these transcripts are rapidly upregulated by the LH surge and that their mature forms remain elevated even after transcriptional decline of the precursor form. This suggests a fairly long half-life for the mature forms. Our *in vitro* experiments demonstrate that the robust induction of *Mirn132* and *Mirn212* seen in response to *in vivo* LH/hCG treatment is likely due to the cAMP cell-signaling cascade. This coincides with the recent observation that cAMP was able to mediate an increase in *Mirn132* levels within rat pheochromocytoma cells (PC12 cells) [61, 74]. Vo et al. [61] also demonstrated several CREB response elements located immediately upstream of each pre-miRNA sequence to be critical for *Mirn132* expression. Our analysis of the pri-miRNA transcript and that of the exonic regions of AK006051, the gene that contains the pri-miRNA transcript for *Mirn132* and *Mirn212* within its first intron, suggests the gene promoter that guides the expression of the mRNA transcript for AK006051 likely plays a significant role in the biogenesis of these miRNAs (data not shown). Vo et al. demonstrated by 5' cap trapping RACE and 3' RACE that the pri-miRNA transcript did not contain a significant open reading frame [61]. Transcriptional regulation of miRNAs is just now beginning to be unraveled, and the transcription of pri-

miRNAs appears to be just as complex as the transcription of protein-encoding genes [42, 45]. This work is a beginning point for future studies concerning the regulation of expression of these two particular miRNAs within ovarian granulosa cells.

To facilitate the identification of proteins and to determine the physiologic role that these LH-regulated miRNAs might be having on granulosa cell function, we used both a computational approach and a knockdown approach. The results of our computational analyses indicate that a large number of 3'UTRs contain *Mirn132/212* seed sequences. Comparison of these lists to the total number of genes present in ovarian granulosa cells after follicular stimulation (i.e., 46 hours of eCG and 1 h post-hCG and 8 h after hCG treatment) yielded a list of 77 transcripts with putative *Mirn132/212* recognition sites. Most of these genes are not specific to ovarian function, and their role in ovarian cell function is unknown. However, several transcripts have been confirmed as *Mirn132/212* targets in other tissues. The MECP2, CTBP1 and p250 GTPase activating-protein (GAP; also known as GRIT within the murine genome) proteins have been shown to regulate neuronal dendritic branching [61, 74]. Interestingly, CTBP1 was recently shown to interact with steroidogenic factor-1 [78] in adrenal cells to modulate its ability to stimulate promoter activity. Our studies indicate that CTBP1 is post-transcriptionally regulated as evidenced by the lack of change in its mRNA levels in response to *in vivo* hCG treatment combined with the pronounced

loss in the protein when miRNA-function blocking LNA oligonucleotides were administered. CTBP1 acts as a co-repressor of nuclear receptor target genes [78]. Therefore, our data suggest that *CtBP1* translation is upregulated in response to LH/hCG/cAMP induction of *Mirn132* and *Mirn212*. In turn, this co-repressor could play key regulatory roles in mediating changes in global gene transcription. We were unable to confirm whether p250GAP (GRIT) and MECP2 were post-transcriptionally regulated due to the lack of suitable antibodies.

In summary, these studies are the first to elucidate the extent of miRNA expression within ovarian granulosa cells and to identify hormonally-regulated miRNAs within periovulatory granulosa cells. The two highly LH-induced miRNAs, *Mirn132* and *Mirn212*, share the same seed sequence and were shown to be transcribed as a single pri-miRNA. The loss of induction of CTBP1 protein synthesis following ablation of functional *Mirn132* and *Mirn212* suggests that these miRNAs may play a key role through indirect regulation of gene transcription in ovarian granulosa cell. Further studies are necessary to identify how these miRNAs mediate changes in expression of CTBP1 as well as to identify the genes downstream of this co-repressor.



## **Chapter 3**

# **Quantitative RT-PCR Methods for Mature MicroRNA Expression Analysis**

### **SUMMARY**

This paper describes two methods to measure expression of mature miRNA levels using qRT-PCR. The first method uses stem-loop RT primers to produce cDNA for specific miRNAs, a technique that our laboratory has modified to increase the number of miRNAs being reverse transcribed within a single RT reaction from one (as suggested by the manufacturer) to five. The second method uses a modified oligo(dT) technique to reverse transcribe all transcripts within an RNA sample; therefore, target miRNA and normalizing mRNA can be analyzed from the same RT reaction. We examined the level of miRNA-132, a miRNA known to be upregulated in granulosa cells following hCG treatment, using both of these methods. Data was normalized to GAPDH or snU6, and evaluated by  $\Delta\Delta C_t$  and standard curve analysis. There was no significant difference ( $P>0.05$ ) in miRNA-132 expression between the stem-loop and modified oligo(dT) RT methods indicating that both are statistically equivalent. However, from a technical point of view, the modified oligo(dT) method was less time consuming and only required a single RT reaction to reverse transcribe both miRNA and mRNA.

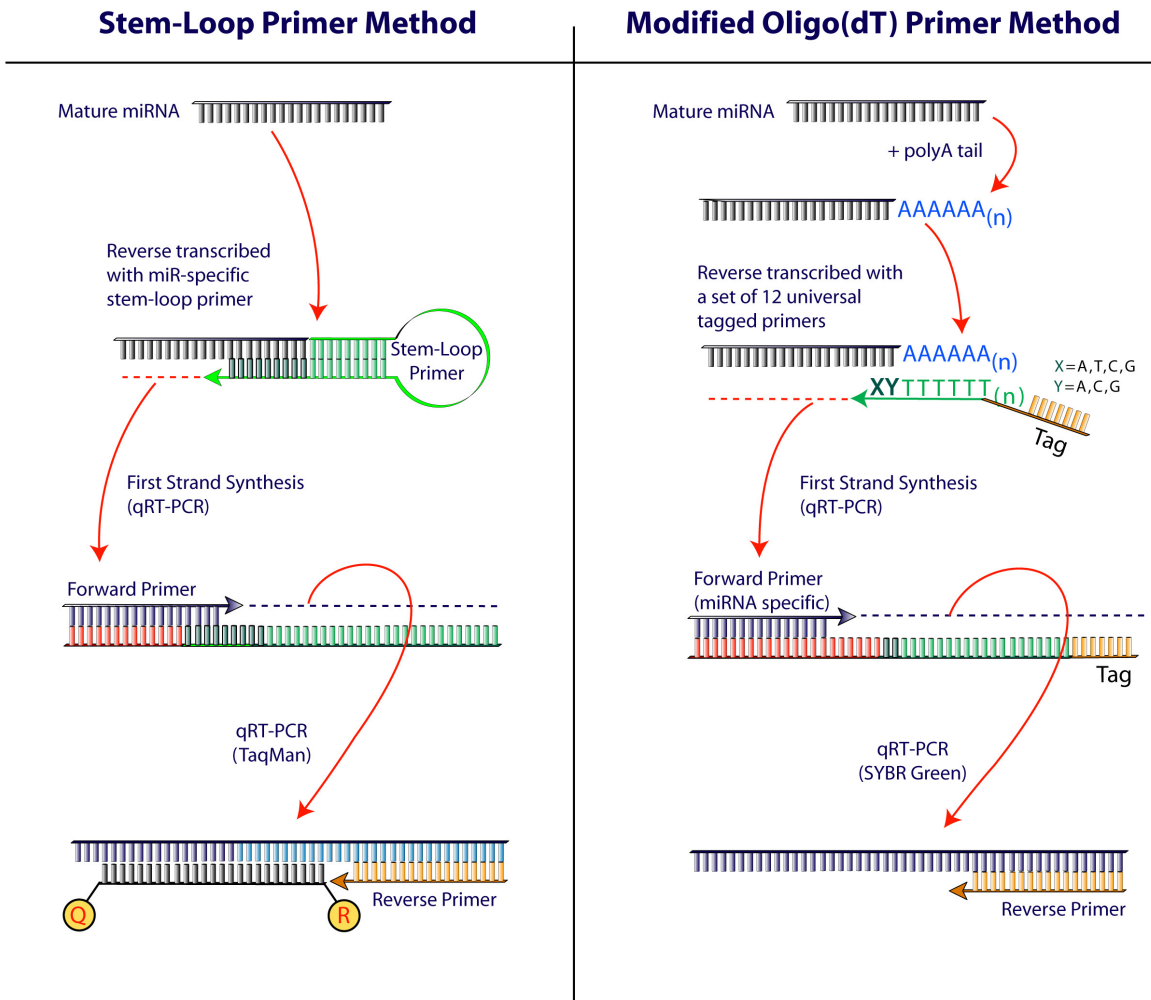
## 1. INTRODUCTION

MicroRNA (miRNA) are endogenous, non-coding single-stranded RNA molecules that are ~22 nucleotides in length [27]. Discovered less than 20 years ago, 695 miRNA have now been identified in the human genome (miRBase Database Version 12.0) and estimates indicate that >1000 exist [46, 101, 102]. MicroRNA have been associated with many biological processes including tissue development, proliferation and differentiation [27, 98, 110], and research links specific miRNA to heart disease and many types of cancer [111-114]. The sequential processing of a primary miRNA transcript (pri-miRNA) to yield a precursor miRNA (pre-miRNA) and ultimately a mature, single stranded miRNA that associates with the RNA-induced silencing complex is well described [27, 33, 40]. MicroRNA microarrays and Northern blotting both provide reliable detection of mature miRNA, but are time consuming, require more starting material, and do not provide the accuracy or precision offered with quantitative RT-PCR (qRT-PCR; [115]).

Analysis of mature miRNA by PCR presents a unique problem as the final length of a miRNA transcript is similar to the primers needed to amplify them. Therefore, expression analysis of mature miRNA has required the development of specialized primers for reverse transcription (RT) or extension of the transcript prior to qRT-PCR. Use of a stem-loop reverse transcriptase primer (described in **Figure 3-1** and **Subheading 3.2.1**; [116]) or a long primer (used in the miR-Q method; [117]) specific to each miRNA is used to

convert total RNA into cDNA. Amplification is then achieved using a primer set containing a primer specific to the mature miRNA of interest and a primer specific to a region of the stem-loop sequence or miR-Q primer [116, 117]. Another approach is to add polyA tails to all transcripts within a sample then reverse transcribe using a set of 12 modified oligo(dT) primers containing a unique sequence tag at the 5' end and two bases at the 3' end (as described in **Figure 3-1** and **Subheading 3.2.3**; [118]). Amplification is then achieved using a PCR primer specific to the miRNA of interest and a primer specific to the tag. Unlike the stem-loop primer RT reaction and the miR-Q method, this RT reaction converts all miRNA and mRNA into cDNA within a single reaction. In this chapter, we discuss modifications that we have made to the stem-loop primer system that allow for simultaneous measurement of up to 5 miRNA. We also compare the stem-loop and polyA tail/modified oligo(dT) RT systems for miRNA-132 expression within murine granulosa using either a standard curve or  $\Delta\Delta C_t$  method of quantification.

**Figure 3-1**



**FIGURE 4-1:** Graphical representation of RT and PCR methods used to analyze the expression of mature miRNAs.

## **2. MATERIALS**

### **2.1 RNA Extraction**

#### **2.1.1 Materials**

1. 19 day old CF-1 mice (20-25g)
2. Disposable latex gloves
3. Pipets (P2, P20, P100, P1000)
4. Sterile, RNase/DNase free filter pipet tips (to dispense volumes between 0.2 -1000 ul)
5. Benchtop centrifuge (refrigerated if possible)
6. Sterile, RNase/DNase free 1.5 ml microcentrifuge tubes
7. -70°C freezer
8. NanoDrop-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE)

#### **2.1.2 Solutions and Reagents**

1. TRIZOL (Invitrogen, #15596-018)
2. Chloroform (Sigma, #C2432-500ML)
3. Glycogen (Invitrogen, #10814-010)
4. Isopropanol
5. 75% Ethanol
6. Ice
7. DEPC water

## 2.2 Reverse Transcription

### 2.2.1 RT Materials

1. Vortexer
2. Benchtop microcentrifuge
3. Thermocycler
4. 0.5 ml sterile PCR tubes with caps
5. Heating block or water bath (capable of reaching 95°C)

### 2.2.2 Stem-Loop Primer Reagents

1. TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, #4366597)
2. hsa-miR-132 Human MicroRNA Assay Kit (Applied Biosystems, #4373143) (*see Note 1*)
3. hsa-miR-212 Human MicroRNA Assay Kit (Applied Biosystems, #4373087)
4. hsa-miR-30b Human MicroRNA Assay Kit (Applied Biosystems, #4373290)
5. dNTPs (Invitrogen, #18427013)
6. Random Primers (Invitrogen, #48190011)
7. MMLV reverse transcriptase (with DTT and 5X RT Buffer; Invitrogen, #28025013)

### 2.2.3 Modified Oligo(dT) Primer Reagents

1. miScript Reverse Transcriptase Kit (Qiagen, #218061)

## 2.3 Quantitative RT-PCR

### 2.3.1 qRT-PCR Materials

1. Applied Biosystems 7900HT Sequence Detection System (may be substituted with other real-time machines)
2. Rainin 250 ul EDP-Plus electronic repeat pipetter
3. ABI Prism 384-well optical reaction plate (Applied Biosystems, #4309849)
4. ABI Prism optical adhesive covers (Applied Biosystems, #4311971)
5. Optical cover applicator tool
6. Centrifuge with swinging bucket optical plate holders

### 2.3.2 Stem-Loop Primer PCR Reagents

1. hsa-miR-132 Human MicroRNA Assay Kit (also used in RT reaction, see **Note 1**)
2. TaqMan Rodent GAPDH Control Reagents (Applied Biosystems, cat. #4308313)
3. TaqMan 2X Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, cat. #4324018)
4. Small nuclear RNA U6 (snU6) primer set - f: ctcgcttcggcagcaca;

r: aacgcttcacgaatttcgt (Integrated DNA Technologies) [119]

5. SYBR Green (Invitrogen, cat. #4309155)

### 2.3.3 Modified Oligo(dT) Primer RT Reagents

1. miScript SYBR Green PCR Kit (Qiagen, cat. #218073)
2. Mm\_miR-132\_1 miScript Primer Assay (Qiagen, cat. #MS00001561)  
(see **Note 2**)
3. TE Buffer, pH 8.0
4. TaqMan Rodent GAPDH Control Reagents (Applied Biosystems, cat. #4308313)
5. Small nuclear RNA U6 (snU6) primer set - f: ctcgcttcggcagcaca; r: aacgcttcacgaatttcgt (Integrated DNA Technologies, Coralville, IA) [119]

## 2.4 Data Analysis

1. Microsoft Office 2003
2. GraphPad Prism (version 4.0)



### 3. METHODS

#### 3.1 RNA Extraction and Dilution

1. Granulosa cells from CF-1 mice (n=3 for each time point) were collected prior to hCG (0h) or 4h and 6h after hCG treatment as previously described in detail [120].
2. Total RNA was isolated from granulosa cells using TRIZOL (1 ml per sample; Invitrogen) with a few modifications to the manufacturer's protocol as described below (see **Note 3**):
  - a. Chloroform (200  $\mu$ l) was added to each tube and tubes were vigorously shaken by hand for 15 seconds before being allowed to rest at room temperature for 5-10 minutes.
  - b. Tubes were centrifuged at 16,100 g for 10 minutes at 4°C.
  - c. Aqueous phase (top layer) was carefully removed from each tube, transferred into a new tube, and then 2  $\mu$ l glycogen (20  $\mu$ g/ $\mu$ l stock) was added to each tube (see **Note 4**). Samples were gently mixed, then 500  $\mu$ l isopropanol was added to each tube.
  - d. Samples were vortexed, incubated at room temperature for 10 minutes, and then centrifuged at 16,100 g for 10 minutes at 4°C.
  - e. Supernatant was poured off (being careful not to lose the pellet), and then the sample was washed with 75% ethanol.
  - f. Tubes were centrifuged at 16,100 g for 10 minutes at 4°C, and the supernatant was then poured off.

g. The RNA pellet was air dried until edges of the pellet began to look clear, then the pellet was rehydrated with 20 ul DEPC water.

Samples were gently pipetted several times to completely dissolve the pellet then immediately placed on ice until RNA quality and concentration determinations were completed.

3. Absorbance and concentration (ng/ul) for each RNA sample was determined with a NanoDrop-1000 spectrophotometer. The ratio of the absorbance at 260 and 280 was used to determine the purity of each sample. Values  $\geq 1.8$  were considered acceptable (see **Note 5**).
4. Aliquots of all 9 RNA samples were diluted with DEPC water to yield 30-50 ul volumes of 50 ng/ul solutions of total RNA. These dilutions were then used in all subsequent RT reactions. Stock and diluted RNA was stored at -70°C until RT reactions were performed.

## 3.2 RT Reactions

### 3.2.1 Stem-Loop Primer RT for Analysis of Multiple (3-5) MicroRNAs

1. Total RNA (250 ng) of the dilution prepared in **Subheading 3.1.4** was reverse transcribed with a combination of three miRNA primers (miRNA-132, miRNA-212, and miRNA-30b; Applied Biosystems; see **Note 6**; [120]). Simultaneous RT reactions for 4 or 5 miRNA are explained in **Note 7**.

2. The master mix for a 3 miRNA RT reaction contained 0.15 ul dNTP mix, 1.0 ul Multiscribe RT enzyme, 1.5 ul 10X RT buffer, 0.19 ul RNase Inhibitor, 1.16 ul nuclease-free water, and 6 ul of RT primers (2 ul of each miRNA-132, miRNA-212 and miRNA-30b RT primers). All reagent volumes are listed as per reaction. The final master mix volume was increased by 10% to prevent a shortage of master mix solution.
3. Master mix (10 ul) and total RNA (5 ul of 50 ng/ul; 250 ng) were pipetted into PCR tubes which were loaded into an thermocycler using the following settings: 16°C for 30 minutes, 42°C for 30 minutes, 85°C for 5 minutes, hold at 4°C.
4. After reverse transcription, samples were diluted 1:15 with DEPC water. Stocks and dilutions were stored at -20°C.

### 3.2.2 Random Primer RT for Normalization of Stem-Loop qRT-PCR

1. Total RNA (1 ug of the 50 ng/ul dilutions made in **Subheading 3.1.4**) was reverse transcribed for GAPDH and snU6 normalization using random primers (Invitrogen; see **Note 8**).
2. The random primer master mix contained 2 ul dNTPs, 0.5 ul random primers (100 ng/ul), and 5.5 ul DEPC water (all reagent volumes are listed as per reaction). The final volume of the master mix was calculated as the sum of the total number of reactions plus 10%.

3. Random primer master mix (8 ul) and total RNA (20 ul of 50 ng/ul) were placed in 1.5 ml microcentrifuge tubes which were then vortexed, briefly spun down, and placed in a 65°C water bath for 5 minutes.
4. Tubes were chilled on ice for 5 minutes immediately after removal from the water bath.
5. A second master mix was prepared containing 2 ul DTT (0.1 M) and 8 ul 5X RT Buffer (volumes are calculated per reaction). Master mix (10 ul) and 2 ul MMLV reverse transcriptase (Invitrogen) were added to each tube (see **Note 9**). Each sample was vortexed and centrifuged briefly then placed in a 37°C water bath for 2 hours.
6. The enzymatic reaction was inactivated by placing tubes in a 72°C water bath for 15 minutes. Reverse transcribed samples were diluted 1:10 with DEPC water. Stocks and 1:10 dilutions were stored at -20°C.

### 3.2.3 Modified Oligo(dT) RT

1. Total RNA (250 ng) from the sample dilutions prepared in **Subheading 3.1.4** was reverse transcribed with the miScript Reverse Transcription Kit (Qiagen; see **Note 10**). All RT reactions were performed according to the manufacturer's instructions.
2. The master mix contained 4 ul miScript RT Buffer (5X), 1 ul miScript reverse transcriptase mix, and 10 ul DEPC water (all reagent volumes

are listed as per reaction). The final volume of the master mix was calculated as the sum of the total number of reactions plus 10%.

3. Master mix (15 ul) and total RNA (5 ul of 50 ng/ul) were placed in 1.5 ml microcentrifuge tubes which were then vortexed, briefly spun down, and placed in a 37°C water bath for one hour.
4. The enzyme reaction was inactivated by incubating all samples on a 95°C heating block for 5 minutes. Reverse transcribed samples were diluted 1:5 with DEPC water. Stocks & dilutions were stored at -20°C.

### **3.3 Quantitative Real Time PCR Reactions**

#### **3.3.1 Generation of Standards for qRT-PCR Assays (see **Note 11**)**

1. Stock cDNA from the **stem-loop RT** reactions (**Subheading 3.2.1, step 4**) was used to generate a standard curve for miRNA-132 qRT-PCR reactions (see **Note 12**).
2. Stock cDNA from the **random primer RT** reactions (**Subheading 3.2.2, step 6**) was used to generate a standard curve for GAPDH and snU6 qRT-PCR reactions (see **Note 12**).
3. Stock cDNA from the **modified oligo(dT) RT** reactions (**Subheading 3.2.3, step 4**) was used to generate a standard curve for miRNA-132, GAPDH, and snU6 qRT-PCR reactions (see **Note 12**).
4. Standard curves were generated by serial dilution of above stock cDNAs as outlined in **Table 3-1** (see **Notes 11 and 12**).

Table 3-1

**TABLE 3-1:** *Serial dilutions for running a standard curve analysis. Initial solution is composed of small amounts from several RT stocks. Each subsequent dilution is made from the previous dilution (i.e. dilution B made from dilution A, dilution C made from dilution B, etc). **It is crucial to vortex each tube well before making the next dilution in the series.***

Dilution	cDNA (RT Product)	Water	Total Volume	Remaining Volume	Dilution Ratio	Arbitrary PCR Value
A.	80	320	400	240	1:5	2000
B.	160 [A]	160	320	240	1:10	1000
C.	80 [B]	320	400	240	1:50	200
D.	160 [C]	160	320	240	1:100	100
E.	80 [D]	320	400	240	1:500	20
F.	160 [E]	160	320	240	1:1000	10
G.	80 [F]	320	400	400	1:5000	2

### 3.3.2 qRT-PCR Using the Stem-Loop RT Product

1. For amplification and detection of mature miRNA-132, qRT-PCR was run in triplicate (10 ul reaction volumes) for standards, samples and negative controls (see **Note 13**) using the miRNA-132 specific primers and probe provided in the hsa-miRNA-132 Human MicroRNA Assay Kit (Applied Biosystems).
2. A master mix consisted of 0.35 ul hsa-miR-132 primer set, 5 ul TaqMan 2X Universal PCR Master Mix, and 3.32 ul DEPC water (volume for each reagent is listed per reaction) and was aliquoted (8.7 ul) into individual wells of a 384-well optical reaction plate using a repeat pipetter. The final volume of the master mix was calculated as the sum of the total number of reactions plus 10%.
3. Samples/standards/negative controls (1.3 ul volume) were then added to each well, the plate sealed with an optical adhesive cover, then centrifuged at 2000 rpm for 2 minutes in a swinging bucket plate holder.
4. The plate was loaded into the Applied Biosystems 7900HT Sequence Detector and run with the following program:
  - a. Stage 1 (TaqMan hot start): 95°C for 10 minutes
  - b. Stage 2 (amplification): 95°C for 15 seconds then 60°C for 1 minute (40 cycles)

### 3.3.3 qRT-PCR for GAPDH and snU6 for Normalization of

#### Stem-Loop qRT-PCR Results

1. For amplification and detection of GAPDH and snU6, qRT-PCR was run in triplicate (10 ul reaction volumes) for standards, samples and negative controls (see **Note 13**) using TaqMan Rodent GAPDH Control Reagents (Applied Biosystems) or the snU6 primer set (Integrated DNA Technologies).
2. A GAPDH master mix was prepared with 0.1 ul of each forward primer, reverse primer, and the probe for rodent GAPDH, 5.0 ul TaqMan Universal PCR Master Mix, and 3.7 ul DEPC water (volume for each reagent is listed per reaction) and was aliquoted (9ul) into individual wells of a 384-well optical reaction plate using a repeat pipetter. The final volume of the master mix was calculated as the sum of the total number of reactions plus 10%.
3. A snU6 master mix was prepared with 90 nM snU6 forward and reverse primers (0.1 ul of 9 uM forward/reverse working solution; see **Note 14**), 5.0 ul SYBR green and 3.9 DEPC water (volume for each reagent is listed per reaction) and was aliquoted (9 ul) into individual wells of a 384-well optical reaction plate using a repeat pipetter. The final volume of the master mix was calculated as the sum of the total number of reactions plus 10%.



4. Samples/standards/negative controls (1 ul volume) were then added to each well, the plate sealed with an optical adhesive cover, then centrifuged at 2000 rpm for 2 minutes in a swinging bucket plate holder.
5. The plate was loaded into the Applied Biosystems 7900HT Sequence Detector and run with the following program:
  - a. Stage 1: 50°C for 2 minutes
  - b. Stage 2: 95°C for 10 minutes
  - c. Stage 3: 95°C for 15 seconds then 60°C for 1 minute (40 cycles)
  - d. Stage 4: 95°C for 15 seconds, 60°C for 15 seconds, then 95°C for 15 seconds

#### 3.3.4 Using Modified Oligo(dT) Primer RT Product

1. For amplification and detection of mature miRNA-132, GAPDH, and snU6, qRT-PCR was run in triplicate (10 ul reaction volumes) for standards, samples and negative controls (see **Note 13**) using the miScript SYBR Green PCR Kit (Qiagen) and the Mm\_miR-132\_1 miScript Primer Assay (Qiagen).
2. A miRNA-132 master mix consisted of 1 ul 10X miScript Universal primer, 1 ul 10X miScript miRNA-132 primer, 5 ul 2X Quantitect SYBR Green PCR Master Mix (see **Note 14**), and 1 ul DEPC water (volumes for each reagent is listed per reaction) and was aliquoted (9 ul) into

individual wells of a 384-well optical reaction plate using a repeat pipetter. The final volume of the master mix was calculated as the sum of the total number of reactions plus 10%.

3. A GAPDH master mix was prepared with 0.1 ul of each forward primer, reverse primer, and the probe for rodent GAPDH, 5.0 ul TaqMan Universal PCR Master Mix, and 3.7 ul DEPC water (volume for each reagent is listed per reaction) and was aliquoted (9 ul) into individual wells of a 384-well optical reaction plate using a repeat pipetter. The final volume of the master mix was calculated as the sum of the total number of reactions plus 10%.
4. A snU6 master mix was prepared with 90 nM snU6 forward and reverse primers (0.1 ul of 9 uM forward/reverse working solution; see **Note 14**), 5.0 ul SYBR green and 3.9 DEPC water (volume for each reagent is listed per reaction) and was aliquoted (9 ul) into individual wells of a 384-well optical reaction plate using a repeat pipetter. The final volume of the master mix was calculated as the sum of the total number of reactions plus 10%.
5. Samples/standards/negative controls (1.3 ul volume) were then added to each well, the plate sealed with an optical adhesive cover, then centrifuged at 2000 rpm for 2 minutes in a swinging bucket plate holder.

6. The plate was loaded into the Applied Biosystems 7900HT Sequence Detector and run with the following program:

a. Stage 1 (TaqMan hot start): 95°C for 15 minutes

b. Stage 2 (40 cycles)

(Denaturation): 94°C for 15 seconds

(Annealing): 55°C for 30 seconds

(Extension): 70°C for 34 seconds (see **Note 15**)

### 3.3.5 Normalization and Analysis Methods (see **Note 16**)

1.  $\Delta\Delta C_t$  Method: This method bypasses the need for a standard curve by simply using the raw qRT-PCR  $C_t$  values. Comparisons can be made between a target of interest and a normalizing mRNA as described in the formula below (see **Note 17**).

$$\text{Fold Change} = 2^{\Delta C_t} = 2^{((\text{Average } C_t \text{ of sample} - \text{Average } C_t \text{ of GAPDH or snU6}) - \Delta C_t^* \text{ of control})}$$

\*  $\Delta C_t = (\text{Average } C_t \text{ of sample} - \text{Average } C_t \text{ of GAPDH or snU6})$ , and the control sample is the control time point, treatment, etc.

2. Standard Curve Method: Arbitrary values for both miRNA and mRNA can be determined by serial diluting cDNA samples produced under the same RT conditions to generate a standard curve. Serial dilutions are then run simultaneously with the samples. Arbitrary values (formula below) determined based on the standard curve (see **Note 11**).

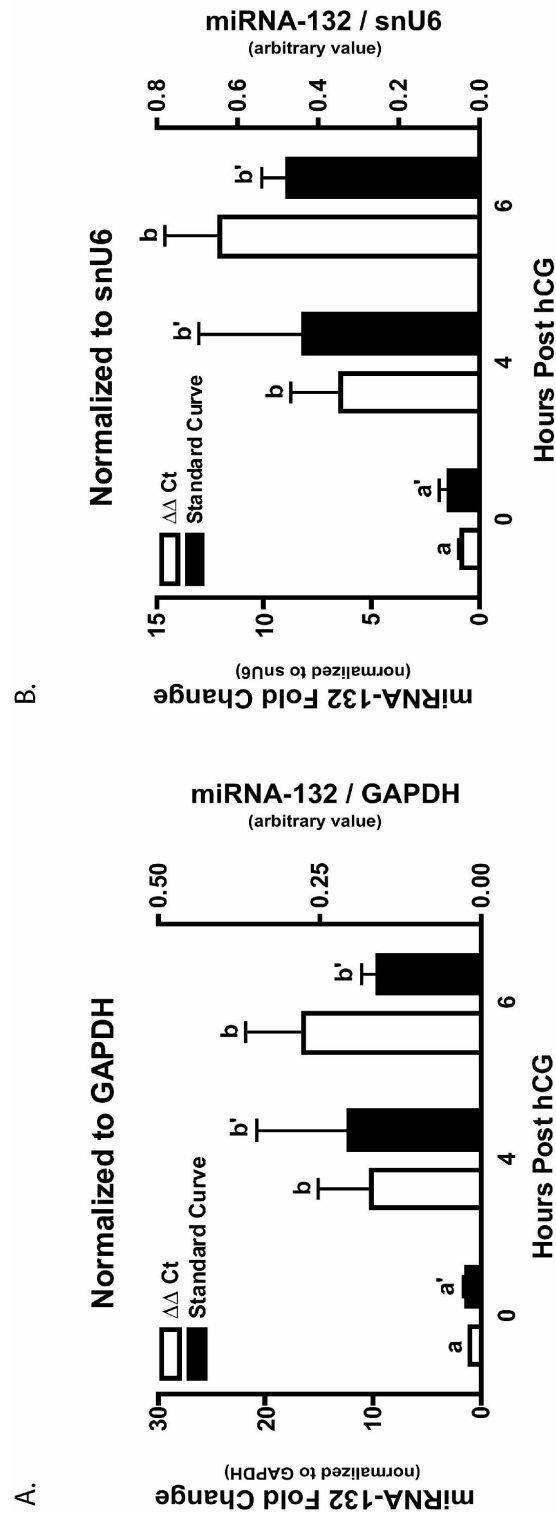
$$\text{Arbitrary Value} = \text{Average of sample} / \text{Average of GAPDH or snU6}$$

3. Comparisons were made by two-way ANOVA (GraphPad Prism) of the stem-loop qRT-PCR (**Figure 3-2**) or modified oligo dT (**Figure 3-3**) results using the  $\Delta\Delta C_t$  and standard curve analyses.
  - a. MicroRNA-132 levels measured by stem-loop qRT-PCR normalized with GAPDH (**Figure 3-2A**) and snU6 (**Figure 3-2B**) were different at 4 and 6 hours post-hCG, but there was no significant difference between the  $\Delta\Delta C_t$  and standard curve analyses.
  - b. MicroRNA-132 levels measured by modified oligo(dT) qRT-PCR normalized with GAPDH (**Figure 3-3A**) and snU6 (**Figure 3-3B**) were different at 4 and 6 hours post-hCG, but there was no significant difference between the  $\Delta\Delta C_t$  and standard curve analyses. In contrast, the snU6 normalization detected a difference ( $P<0.05$ ) between the 4 and 6 h time points using the  $\Delta\Delta C_t$  analysis that was not seen with the other methods/analyses.
4. Side-by-side comparisons were made by two-way ANOVA (GraphPad Prism) of stem-loop versus modified oligo(dT) qRT-PCR  $\Delta\Delta C_t$  analyzed data normalized with either GAPDH (**Figure 3-4A**) or snU6 (**Figure 3-4B**; see **Note 18**).

Method [stem-loop versus modified oligo (dT)] of miRNA-132 quantification was not different ( $P>0.05$ ) for either GAPDH or snU6 normalized data nor was there a method by hour interaction. We did observe a trend ( $P=0.07$ ) for a method [stem-loop versus modified oligo (dT)] difference but only in the  $\Delta\Delta C_t$

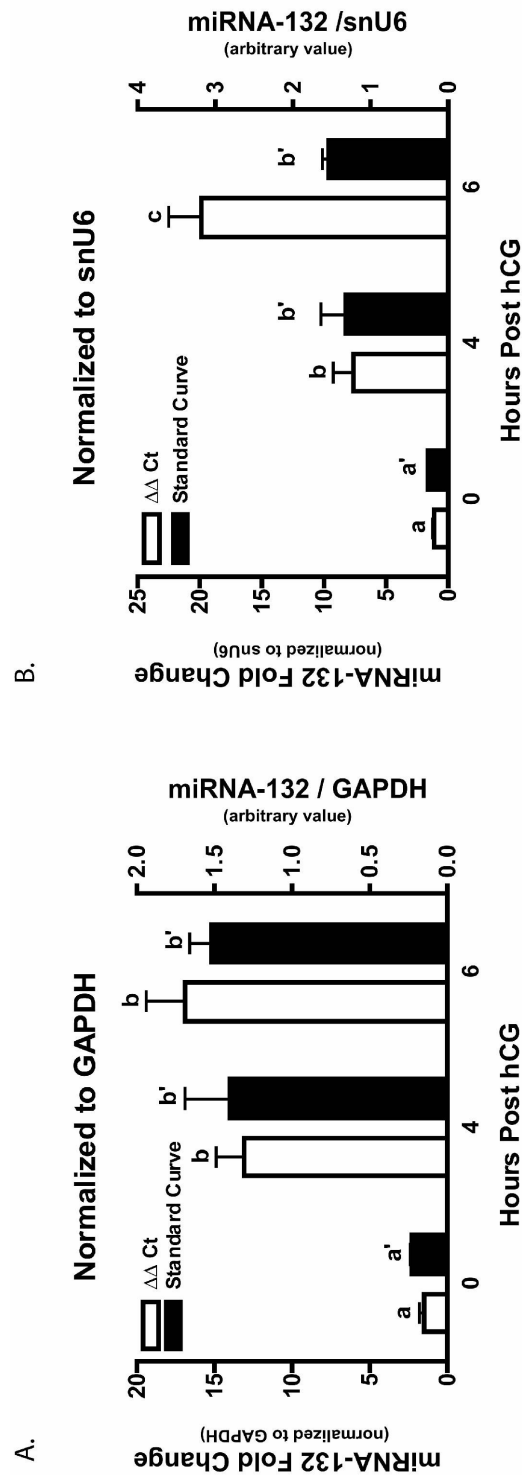
analysis. Although no differences between the two methods were observed, the standard error values did appear to be decreased in samples reverse transcribed using the modified oligo(dT) RT system.

Figure 3-2



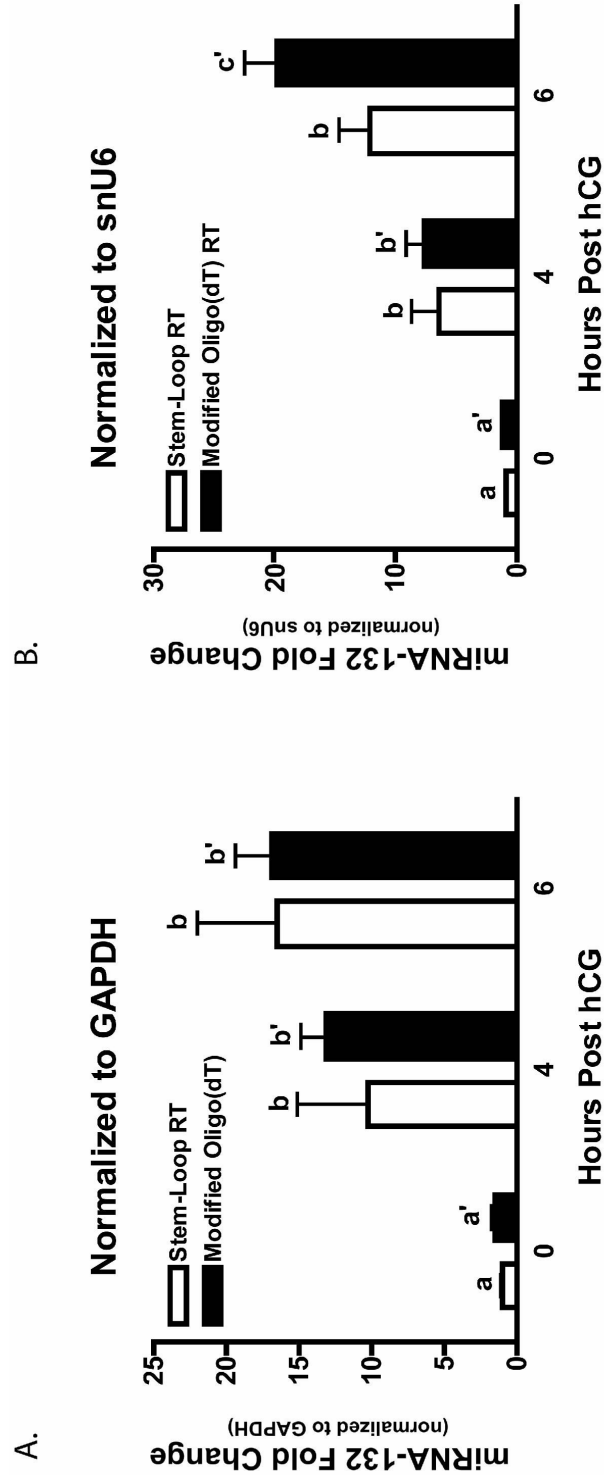
**FIGURE 3-2: Stem-Loop qRT-PCR Method** - Comparison of mural granulosa cell miRNA-132 levels before (0 h) and 4 and 6 h after hCG using the  $\Delta\Delta$ Ct and standard curve analysis for stem-loop qRT-PCR method. Mature miRNA-132 levels were normalized to GAPDH (A) or snU6 (B). <sup>a,a'</sup> & <sup>a',b'</sup> Means +/- SEM within an analysis group ( $\Delta\Delta$ Ct or standard curve, respectively) with different superscripts are significantly ( $P<0.05$ ) different.

Figure 3-3



**FIGURE 3-3: Modified Oligo(dT) qRT-PCR Method** - Comparison of mural granulosa cell miRNA-132 levels before (0 h) and 4 and 6 h after hCG using the  $\Delta\Delta$ Ct and standard curve analysis for modified oligo(dT) qRT-PCR method. Mature miRNA-132 levels were normalized to GAPDH (A) or snU6 (B). <sup>a,b</sup> & <sup>a',b'</sup> Means  $\pm$  SEM within an analysis group ( $\Delta\Delta$ Ct or standard curve, respectively) with different superscripts are significantly ( $P < 0.05$ ) different.

Figure 3-4



**FIGURE 3-4:** Comparison of stem-loop and modified oligo(dT) qRT-PCR methods for quantification of mural granulosa cell miRNA-132 levels using  $\Delta\Delta Ct$  analysis. Mature miRNA-132 levels were normalized to GAPDH (**A**) and method by hour interactions were not significant, while day was significant ( $P<0.05$ ). <sup>a,b,c</sup> & <sup>a',b'</sup> Means  $\pm$  SEM within an analysis group ( $\Delta\Delta Ct$  or standard curve, respectively) with different superscripts are significantly ( $P<0.05$ ) different. We observed a trend ( $P=0.07$ ) for a method difference in the GAPDH comparison.



#### 4. NOTES

1. The mature forms of both human and murine miRNA-132, 212 and 30b are 100% homologous; therefore, the human primer sets can be used to analyze the expression of these mouse miRNA. Data analysis within this paper focused on miRNA-132 expression; therefore, qRT-PCR data for miRNA-212 and miRNA-30b are not shown.
2. Individual miRNA specific qRT-PCR primers can be designed and substituted for miScript primers within the PCR section of the modified oligo(dT) protocol (Qiagen). We have designed our own miRNA-132 specific primers and observed similar results as provided with primers from the kit. It is important to test designed primers for specificity and efficiency; in this case, this was done by comparing expression levels produced by the designed primer to those produced by the kit primer.
3. Several kits and reagents are used within the methods described in this paper. These products are all provided with extensive protocols that include many tips and troubleshooting suggestions.
4. Glycogen can be added to samples during the RNA isolation process to act as a carrier of nucleic acids and will not interfere with RT or PCR reactions when used at concentrations lower than 250 ug/ml. For our purposes, samples were isolated with 40 ug/ml glycogen, a concentration that provides higher RNA yields while remaining well below maximum levels.

5. RNA samples can be contaminated with proteins, salts and DNA. To prevent these types of contamination, extra caution should be taken when transferring the aqueous layer during the isolation process. The 260/280 ratio should be at least 1.8 - 2.0 with lower values suggesting high levels of protein within a sample; while a 260/230 ratio below 1.8 can indicate the presence of organic contaminants including Trizol. Although the NanoDrop spectrophotometer is a highly sensitive instrument capable of very accurately measuring these ratios as well as the RNA concentration using only 1  $\mu$ l of the sample, any standard spectrophotometer can be used to determine absorbance and concentration values.
6. The instructions for the TaqMan MicroRNA Reverse Transcription kit suggest a starting amount between 1 and 10 ng of total RNA per 15  $\mu$ l reaction. Following the PCR reaction, this amount of RNA consistently yielded high Ct values (i.e. low transcript levels) for miRNA within granulosa cells; therefore, we increased the starting amount of total RNA to improve the RT reaction and thus produce more robust qRT-PCR results.
7. The stem-loop RT method provides a quick, reliable way to measure the expression of mature miRNA using a relatively small starting amount of RNA. The manufacturer's protocol provides instructions to reverse transcribe one specific miRNA per RT reaction. Our laboratory

has determined that it is possible to combine up to 5 RT primers in one reaction [120]. To do this, adjustments have been made to the water volume, the primer amount and/or the total reaction volume. For the stem-loop RT reactions described in this chapter, three specific primers were combined (data for miR-132, one of the three miRNA is shown). The total volume of the reaction (15 ul reaction) follows the manufacturer's protocol, although the volume of water was adjusted to 1.16 ul and the amount of each primer was reduced to 2.0 ul of each (compared to 3 ul recommended by the manufacturer). A combination of either four or five RT primers requires the volume of the RT reaction to be increased from 15 ul to 20ul. Volumes of all the reagents change accordingly [i.e., 0.2 ul dNTP mix, 1.5 ul Multiscribe, 2.0 ul 10X RT buffer, 0.25 ul RNase inhibitor, 3.05 ul (for 4 primers) or 1.05 ul (for 5 primers) DEPC water, and 2 ul of each primer]. Each RT reaction consists of 15 ul of master mix plus 5 ul RNA (50 ng/ul). **It is important that expression levels of miRNA amplified in a combination RT reaction should first be compared to levels produced when each miRNA is reverse transcribed alone to make sure certain primer combinations do not interfere with each other and affect the PCR reaction.**

8. The TaqMan MicroRNA Reverse Transcription kit is designed to reverse transcribe only mature miRNA transcripts, therefore

normalization to a non-changing (i.e. constitutively expressed RNA) is done by performing two separate RT reactions, one for the miRNA and one for the normalizer (i.e. GAPDH or snU6) as described in this chapter. Of note, we have demonstrated that simultaneous reverse transcription of snU6 within the stem-loop primer RT reaction (data not shown) provides the same results as compared to two separate RT reactions (shown in **Figure 3-2**).

9. Remove MMLV reverse transcriptase from freezer right before needed and return to the freezer immediately after using it.
10. One of the added benefits of the miScript Reverse Transcription Kit (shown in **Figure 3-1**) is that both oligo(dT) primers and random primers are contained within the RT buffer. Therefore, miRNA and mRNA are reverse transcribed within the same tube allowing both miRNA and normalizing mRNA from a sample to be analyzed following a single RT reaction rather than two separate RT reactions as required in the stem-loop RT and miR-Q RT methods. The miScript Reverse Transcription Kit is optimized for starting amounts between 10 pg and 1 ug of total RNA.
11. Quantitative RT-PCR is only quantitative when the amount of cDNA is determined during the exponential doubling phase. Fluorescence is measured to establish threshold cycle (Ct) values which represent the amount of product amplified at a given point in the reaction. When

more template is available at the beginning of the reaction, a fewer number of cycles is necessary for the fluorescent signal to measure above the background level. Therefore, diluting cDNA will cause an increase in the number of cycles needed to reach a given fluorescence value. This concept can be used to establish arbitrary fluorescence values by creating a standard curve from a serial dilution for a given sample set. To do this, an initial stock solution is made from small volumes of RT stocks *prepared under the same RT conditions*.

Arbitrary values can be assigned to each dilution based on the fold differences between each of the dilutions in the series, and a relative value for an unknown can be extrapolated by comparing its signal to standard curve [115].

12. The standard curve should be made from samples that are known or predicted to have high transcript levels to ensure that the unknown sample values will fall within the range of arbitrary values generated by the curve.
13. Negative controls include samples that were not reverse transcribed and water blanks. The absence of fluorescence in non-reverse transcribed samples indicates the absence of DNA contamination. We typically run non-reverse transcribed negative controls for a single miRNA and GAPDH/actin for each sample dilution. If the results are negative for genomic contamination for these genes, we make the

assumption that the sample is negative for all DNA contamination. However, if the laboratory has used transfection agents, like pre-miRNA, siRNA or expression plasmids for specific genes, a negative control should always be run for that specific transcript simultaneously with the reverse transcribed sample, as plasmid contamination is a major problem for qRT-PCR assays. Water blanks are included to confirm the absence of primer dimers (in the case of SYBR green qRT-PCR) and to confirm that contamination of the PCR product has not occurred within the space used to set up the qRT-PCR assays.

14. PCR primer sets designed without a probe (i.e. snU6 described above) rely on SYBR green detection. This dye binds non-specifically to double stranded DNA causing the fluorescence signal to increase as the amount of double stranded DNA increases. This method of measuring fluorescence is vulnerable to primer dimers; therefore, a primer check should be performed to determine the highest concentration at which the primers can be used without forming dimers. The presence of primer dimers can be determined from dissociation curve analysis. The melting points for a given primer set should all be the same, while the shorter sequence formed by primer dimers will melt at a lower temperature which is indicated by a peak shift. It is not necessary to perform a primer check when primer sets

provided within a kit are used (miScript Primer Assay, Qiagen), as the company will provide optimized conditions for each reagent.

15. The extension time recommended by the miScript System Handbook (30 seconds) is optimized for the ABI PRISM 7000 and an adjustment to 34 seconds is suggested when using Applied Biosystems 7300 and 7500 Systems. As an Applied Biosystems 7900HT Sequence Detector was used for amplification of our samples, we chose a 34 second extension period.
16. It is not necessary to evaluate data with *both*  $\Delta\Delta C_t$  and standard curve analyses.
17. Normalization is performed to account for variation between unknown samples and can be achieved by comparing fluorescent values from a gene of interest to those from a gene expressed at a constant level within a tissue/cell. The  $\Delta\Delta C_t$  data analysis method can be used to determine a relative fold-change between samples, but the calculations used to determine these values depend on the assumption that the PCR reaction is equally efficient for both the transcript of interest and that of the normalizer (GAPDH/snU6; [115]).
18. As there was no significant difference between the  $\Delta\Delta C_t$  and the standard curve methods (**Figures 3-2 and 3-3**), only the  $\Delta\Delta C_t$  analysis is shown in the side-by-side comparison of the two RT methods.

## **Chapter 4**

### **Transcriptional Regulation of MicroRNA-132/212 Expression in Murine Periovarian Granulosa Cells**

#### **ABSTRACT**

MicroRNAs (miRNA) are highly conserved, 19-21 nucleotide non-coding RNAs that bind to the 3'-untranslated region of specific target mRNAs to regulate translation. We have recently shown that the mature forms of miRNA-132 and miRNA-212 are rapidly (< 2 hours post-hCG) and dramatically upregulated (34-fold) in periovarian mural granulosa cells. Moreover, *in vitro* studies indicated that 8-Br-cAMP treatment of granulosa cells mimicked LH-induced *in vivo* expression of miRNA-132 and 212. These two microRNAs share the same seed sequence (a region considered to be important in target recognition), and they are located within 203 bases of each other in the intron of an uncharacterized gene (AK006051). This study sets out to determine the identity of the nascent transcript(s) for miRNA-132 and 212 within periovarian granulosa cells and the mechanisms regulating the expression of these miRNAs. No significant differences were found in the proteins of the miRNA biogenesis pathway (Drosha, DGCR8, Exportin-5 and Dicer) over a 12-hr time period following hCG treatment in 19 day old female CF-1 mice when analyzed by quantitative RT-PCR (qRT-PCR), suggesting that the induction of mature miRNA-132 and 212 occurs as a result of increased expression of the miRNA transcript rather than increased miRNA

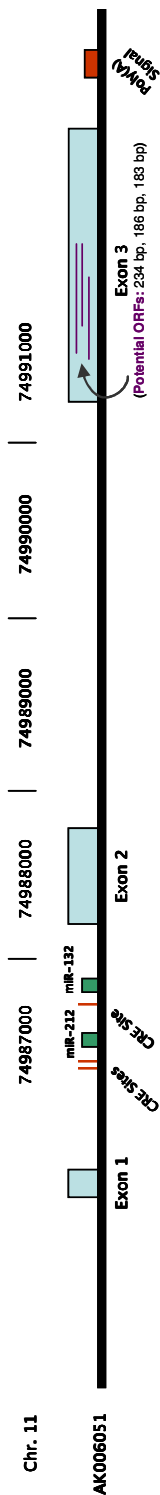


processing. To determine whether these miRNA are co-expressed as a single pri-miRNA transcript, a 477 bp pri-miRNA-212/132 transcript was amplified with primers designed to span a region 5' to pre-miR-212 through the 3' region of pre-miR-132. The single pri-miRNA transcript and similar expression profiles of pre-miRNA-132 and 212 suggest that these two miRNA may be regulated by a common promoter in mural granulosa cells. Interestingly, several CRE sites located 5' to the pre-miRNA sequences have been implicated in the regulation of miRNA-132 and 212 expression within the neuronal cell line. As both miRNA-132 and 212 pre-miRNA sequences reside within the first intron of a gene (AK006051) coding a mRNA transcript of unknown function, qRT-PCR primers were designed to span the junction between the first and second exons of this gene. Despite the fact that these exons do not encode a significant open reading frame, the expression of exon 1 / 2 and 2 / 3 transcripts were shown to dramatically increased over the 12 hour time course. Our studies demonstrate that miRNA-132 and miRNA-212 are coordinately regulated by LH/cAMP. Further studies are necessary to fully understand the role of the intronic CRE sites and their interaction with the AK006051 promoter.

## INTRODUCTION

MicroRNA (miRNA) are highly conserved, 19-21 nucleotide non-coding RNAs that bind to the 3'-untranslated region of specific target mRNAs to regulate translation. We have recently shown that the mature forms of miRNA-132 and miRNA-212 are rapidly (< 2 hours post-hCG) and dramatically upregulated (34-fold) in periovulatory mural granulosa cells [120]. Moreover, *in vitro* studies indicated that 8-Br-cAMP treatment of granulosa cells mimicked LH-induced *in vivo* expression of miRNA-132 and 212. These two microRNAs share the same seed sequence (a region considered to be important in mRNA target recognition), and their pre-forms are located within 203 bases of each other within the intron of an uncharacterized gene (AK006051; **Figure 4-1**). Additionally, several CRE sites located 5' to the pre-miRNA sequences have been implicated in the regulation of miRNA-132 and 212 expression within nerve cells [61]. Interestingly, previous studies within a rat neuronal cell line have reported AK006051 to produce a non-coding transcript with no significant open reading frame (ORF; [61]). The objectives of this study were to first identify nascent transcript(s) for miRNA-132 and 212, then begin to identify regulatory mechanisms involved with miRNA-132/212 expression.

Figure 4-1



**FIGURE 4-1:** AK006051 contains three exons and two introns. The pre-miRNA sequences for both miRNA-132 and 212 both reside with the first intronic region of this gene.

## METHODS AND MATERIALS

### *Animals and Granulosa Cell Isolation*

Nineteen-day old CF-1 female mice were injected i.p. with 5IU eCG to induce follicular stimulation. After 46 hours, the ovaries were either collected (0 hour time point) or the mice were injected i.p. with 5IU of hCG and ovaries collected at 1, 2, 4, 6, 8 and 12 hours post-hCG. Periovulatory follicles were punctured with a 30-gauge needle and mural granulosa cells were collected into PBS. RNA from these granulosa cells was extracted using TRI Reagent as per the manufacture's protocol (Sigma, St. Louis, MO).

### *Quantitative RT-PCR for Messenger RNAs and Pri-miRNAs*

Intron spanning primers for AK006051 (exons 1 & 2 or 2 & 3) were designed using the Primer Express 3.0 software (Applied Biosystems), and quantitative RT-PCR (qRT-PCR) was conducted on a 7900 HT Sequence Detection System (Applied Biosystems). Each sample was run in triplicate, and the  $\Delta\Delta C_t$  method was used to calculate relative fold-change values between samples. To confirm the existence of a single pri-miRNA-132/212 that it is transcriptionally upregulated in response to LH, a qRT-PCR primer set was designed to amplify a 349 base pair region spanning from the 5' end of the pre-miRNA-212 through the 3' end of the pre-miRNA-132 (**Figure 4-2**). qRT-PCR to analyze the temporal expression of mature murine miRNA-132 was performed using a human RT kit and a miRNA assay kit (Applied

Biosystems). AK006051 expression was normalized to GAPDH expression for all samples using the Rodent GAPDH Taqman primers and probe (Applied



**FIGURE 4-2:** Primer design for qRT-PCR amplification of pri-miRNA-132/212. Dotted lines indicate the positions of the forward and reverse primers. Biosystems).

#### *Amplification and Sequencing of AK006051*

Murine mural granulosa cells were collected four hours post-hCG and total RNA isolated as described above. The 5` end of the AK006051 gene was identified using a FirstChoice® RLM-RACE (RNA Ligase Mediated Rapid Amplification of cDNA Ends) kit (Ambion) in combination with two gene specific primers designed to amplify a region through the 5` end of the third exon.

## RESULTS

### *Temporal Expression of Pri-miR-132/212 in Granulosa Cells In Vivo*

The in vivo expression pattern for pri-miR-132/212 within mural granulosa cells was determined by qRT-PCR. Analysis of pri-miRNA levels over the 12 hour periovulatory period indicated the existence of a single pri-miR-132/212 transcript. This transcript was significantly upregulated at 2 and 4 hours post-hCG then returned to basal levels by the 6 hour time point (**Figure 4-3A**). The overall expression pattern for pri-miR-132/212 appeared to precede the expression levels seen within the mature forms of these miRNA (mature miRNA-132 shown inset, **Figure 4-3A**). Validation of the qRT-PCR product done by gel electrophoresis showed an ~349 base pair band indicating that the primers amplified a product of the expected size (**Figure 4-3B**). Sequencing of the qRT-PCR product confirmed amplification of the expected sequence fragment (data not shown).

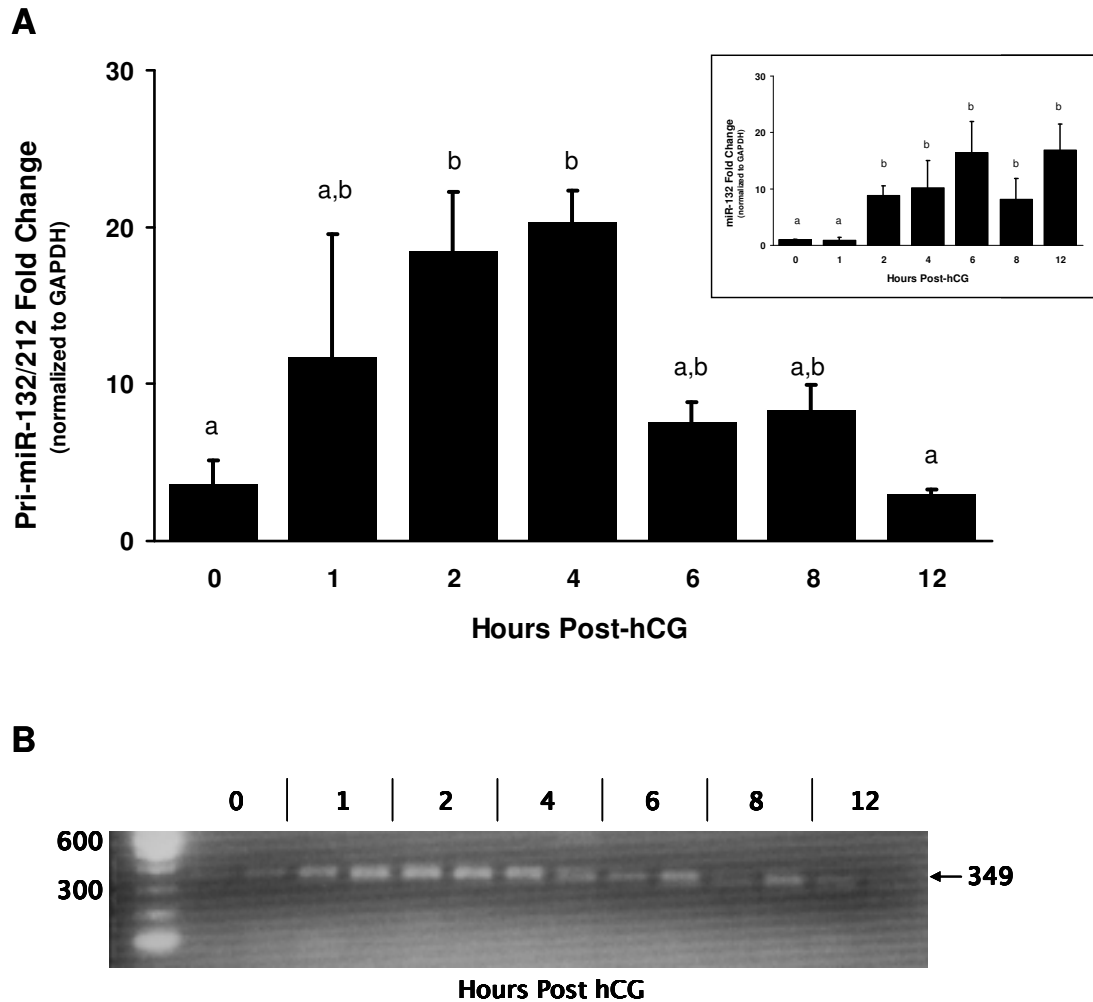
### *Temporal Expression of AK006051 in Granulosa Cells In Vivo*

The in vivo expression pattern for AK006051 was determined by qRT-PCR. Analysis of this gene using primers designed to span the junction between the first and second exons indicated this transcript to be upregulated by 1 hour post-hCG (**Figure 4-4A**). Expression levels showed a large increase over basal levels at 2 and 4 hours post-hCG (~700 and 600-fold respectively) and continued to be upregulated through the 12 hour time point.

Analysis of AK006051 using a primer set designed to span the second and third exons displayed a similar pattern as previously described with expression levels upregulated by 1 hour post-hCG, peaking at 2 hours (~300-fold) post hCG before dropping slightly at 4 hours (~200-fold) post-hCG (**Figure 4-4B**). Transcript levels continued to be upregulated compared to basal levels through the 12 hour time point.

RACE analysis of the 5' end of the AK006051 gene in murine granulosa cells indicated two sequencing differences (n=1) compared to the UCSC Genome Browser database: 1) Exon 1 was missing 'GGG' at the 5' end and 2) Exon 3 had a single nucleotide change (T → C) at the 29th base. No other alternative transcripts were found.

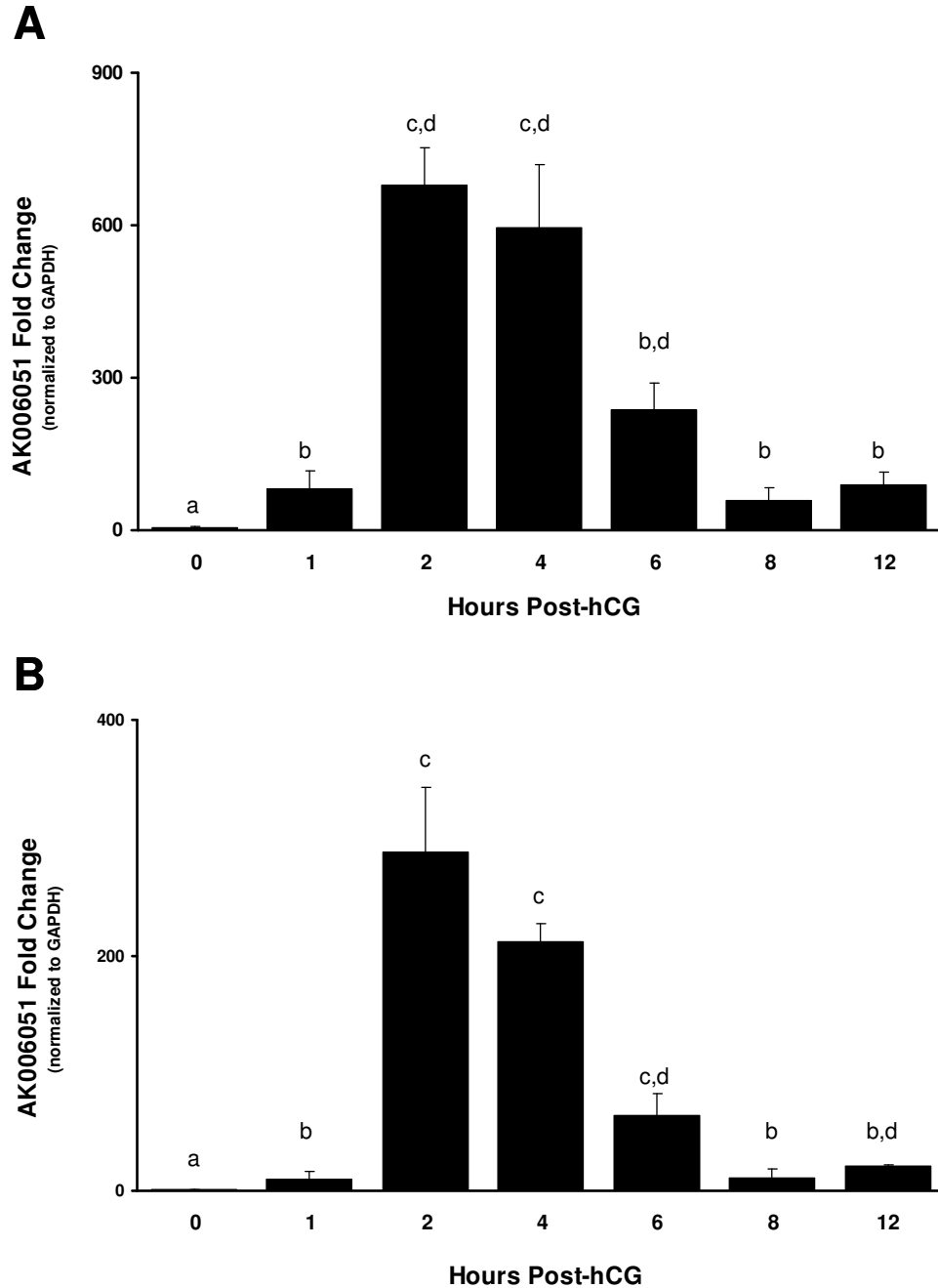
Figure 4-3



**FIGURE 4-3:** qRT-PCR analysis of pri-miR-132/212 transcript levels **(A)** in mural granulosa cells collected from mice following *in vivo* treatment with eCG for 46 hours (i.e., 0 hour) and eCG + hCG for 1 to 12 hours post-hCG. mRNA levels were normalized to GAPDH (mature miRNA-132 expression is shown in the inset). **(B)** qRT-PCR product analyzed by gel electrophoresis. Means  $\pm$  SEM with unlike superscripts (a,b) are different ( $P < 0.05$ ).



Figure 4-4



**FIGURE 4-4:** qRT-PCR analysis of AK006051 (**A**) Exons 1/2 and (**B**) Exons 2/3 transcript levels in mural granulosa cells collected from mice following *in vivo* treatment of eCG for 46 hours (i.e., 0 hour) and eCG + hCG for 1 to 12 hours post-hCG. mRNA levels were normalized to GAPDH. Means  $\pm$  SEM with unlike superscripts (a,b,c,d) are different ( $P < 0.05$ ).

## DISCUSSION

The factors and events involved with regulating transcription of miRNA are largely unknown. Sequences coding for miRNA can be found in intergenic regions of the genome as well as within exons and introns of known genes [121]. The diverse nature of their locations suggests that more than one mechanism may be involved with miRNA transcription. It has been speculated that transcription of intergenic miRNA occurs via promoters specific to these particular miRNA, and it remains to be determined whether intronic miRNA have their own promoter sequences [122-124] or if they rely upon promoters found in the gene within which they are located [124-126]. The dramatic increase in AK006051 expression following the LH surge, a finding that contrasts with that seen by others in neuronal cells [61], would suggest that transcription of miRNA-132 and 212 may be regulated by this gene.

Our studies demonstrate that miRNA-132 and 212 are coordinately regulated by LH/cAMP to produce a single pri-miRNA-132/212 transcript and that the gene within which they reside is dramatically upregulated by 2 hours post-hCG. Further studies are necessary to fully understand the role of the intronic CRE sites and their interaction with the AK006051 promoter. The data shown within this chapter represent a starting place for studying the transcriptional regulation of miRNA-132 and 212, an event that appears to be regulated by the LH surge within periovulatory mouse granulosa cells.

## **Chapter 5**

### **Conclusions and Future Directions**

In the ovary, the follicular granulosa cells provide the proper environment (factors) necessary for development of a competent oocyte in response to the pituitary hormones, follicle stimulating hormone and luteinizing hormone (LH). Moreover, ovulation of the egg and the subsequent luteinization of granulosa cells are dependent on the surge of LH [9, 62]. Although much is known about how the genes involved with ovulation and luteinization are regulated from a transcriptional level, very little is known about how these transcripts are post-transcriptionally regulated prior to being translated into a protein. Recently, microRNA (miRNA) have been implicated as key players in post-transcriptional gene regulation. Hundreds of unique miRNA genes exist in mammals, and each miRNA is postulated to be capable of binding to multiple mRNAs, thereby allowing them to act as key regulators in a variety of cell processes including differentiation, proliferation and death [127, 128]. Following the LH surge, periovulatory granulosa cells undergo a rapid and marked differentiation as they convert from proliferative, estrogen producing cells to terminally differentiated progesterone producing luteal cells. Given the ability of miRNAs to silence the translation of multiple messages and the known rapid differentiation process that occurs in periovulatory

follicles in response to the LH surge, we speculate that miRNA may play a key role in ovarian function.

As many miRNAs have been shown in other systems to be involved with proliferation and cell differentiation, and LH induces differentiation in granulosa cells, we are interested in examining the role miRNA play in regulating target mRNA(s) following the LH surge. To this end, we have used microarray analysis to identify 212 miRNA expressed in murine granulosa cells at 0 and 4 hour post-hCG, and we have demonstrated this to be a robust method for identification miRNA expression in the ovary as compared to cloning techniques used by others [108].

Additionally, we have identified miRNA-132 and -212 as being significantly upregulated by LH in granulosa cells. MicroRNA-132 was previously implicated in regulating cAMP-induced differentiation of neuronal cells [61]. This suggests that miRNA-132 may play a similar role in granulosa cell differentiation as it is also a process mediated by cAMP/PKA signaling. Interestingly, miRNA-132 and 212 have identical seed sequences suggesting they may target similar mRNA(s). Using bioinformatics analysis tools, a comparison of potential miRNA-132 and 212 targets to transcripts expressed within periovulatory granulosa cells yielded a list of 77 potential mRNA targets. Western blot analysis of CTBP1, a putative target of miRNA-132 and 212, indicated a decrease in protein levels following knockdown of both miRNA-132 and 212. CTBP1 has been recently identified as a co-repressor of

nuclear receptor genes known to regulate the expression of CYP17 in the adrenal cortex [78]. Although CYP17 is not expressed within GCs, CTBP1 has been shown to interact with steroidogenic factor-1 (SF-1) which is expressed by granulosa cells and is associated with regulating genes associated with steroidogenesis and maintaining healthy ovarian function [129]. This suggests a possible functional role for miRNA-132 in the regulation of CTBP1 and the indirect regulation of SF-1 expression in granulosa cells.

The increase in expression of miRNA-132 and 212 following the LH surge is the result of an unknown mechanism, but we hypothesize that it is a regulated event involving key transcription factors and regulatory elements. As previous studies have shown co-expression of clustered miRNAs [130, 131], we were not surprised that qRT-PCR analysis indicated these two miRNAs to be expressed as a single pri-miRNA transcript. This data would suggest that miRNA-132 and 212 are regulated by the same promoter. As there are CRE sequences upstream of both of these two particular miRNAs, the logical place to begin studying their transcriptional regulation is by examining the transcription factor CREB. Initial experiments should be designed to determine whether LH-induced expression of miRNA-132 and 212 is a direct result of CREB binding to CRE sites upstream of the miRNA sequence and to determine whether CREB preferentially binds at CRE sites upstream of miRNA-132 and 212 following LH-induction.

Interestingly, previous studies have shown that while intronic miRNAs can act as independent transcriptional units [122-124], others rely on the transcription of their host gene [27, 124-126]. The pre-forms of miRNA-132 and 212 both fall within the first intron of the AK006051 gene. Therefore, it is entirely possible that transcription of these two miRNA could be regulated by the promoter of this gene rather than being under the control of their own promoter. Initial qRT-PCR analysis of AK006051 mRNA levels indicated that this gene is dramatically upregulated following the LH surge. This would suggest a possible ovarian function; therefore, future studies should include experiments designed to examine any phenotypic changes that may occur in granulosa cells following the over-expression of this transcript.

Although it is tempting to focus on CREB's role in regulating miRNA-132 and 212 expression as the PKA pathway is known to be the main signal transduction pathway induced by the LH surge [10, 62], it is possible that other cAMP-responsive transcription factors like ATF-1, NF- $\kappa$ B, and CEBP- $\beta$  could potentially be involved. With this in mind, it may be relevant to design assays to analyze these transcription factors as well. Moreover, although miRNA-132 and 212 are induced following the LH surge and CRE sites are positioned near them, these sites may not be involved with promoting the transcription of these miRNA. Alternative experiments may focus on other DNA elements like Sp1, AP1 and GATA sites that could potentially be involved with the expression of these specific miRNAs.

In conclusion, we have identified and have begun to characterize two of the most highly upregulated miRNA with periovulatory granulosa cells. Understanding how these two miRNA regulate translation as well as how they are transcriptionally regulated will provide novel information about miRNA regulatory mechanism(s) and will begin to shed light on the involvement of miRNA within the processes of ovulation and luteinization.

## **Chapter 6**

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